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(54) Title: BETA-AMYLOID TOXICITY

(57) Abstract

The present invention relates to methods and compositions for visualization of the toxic effects of transgenes *in vivo*. In particular, the present invention provides methods and compositions for the production and use of transgenic, including dually transgenic, *Caenorhabditis elegans* for visualization of the toxic effects of β-amyloid accumulation *in vivo*.

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BETA-AMYLOID TOXICITY

FIELD OF THE INVENTION

The present invention is in the field of medicine and molecular biology. In particular, the invention provides transgenic non-human animals in which expression of a reporter gene is induced by a toxic transgene. The invention also provides methods and compositions for *in vivo* visualization of the toxicity associated with toxic proteins. In addition, the present invention provides methods and compositions for the production and use of transgenic non-human animals for screening candidate drugs to assess and treat toxicity.

BACKGROUND OF THE INVENTION

Investigation of the molecular mechanisms of various diseases has suggested that many pathologies are caused by expression of proteins that are immediately or gradually deleterious to the cells where they are expressed. Among these diseases are Alzheimer's disease, prion diseases, Huntington's disease, and amyotrophic lateral sclerosis. Several of these diseases are thought to result from aberrant folding of proteins, which results in the accumulation of toxic proteins or protein aggregates.

Alzheimer's disease is associated with the majority of dementia cases in the United States, with an estimated 2 million people afflicted with the disease, and a mortality rate of approximately 100,000 people per year (*See*, R.W.P. Cutler, "Degenerative and Hereditary Diseases," *in Medicine*, Scientific American, New York, (1988), pages 11 (IV):1-13; and R. Katzman (1986) *N. Engl. J. Med.* **314**:964). It has been estimated that the total cost for nursing home care alone of Alzheimer's patients exceeds $13 billion/year (*See*, M.M. Heckler (1985) *Am. Psychol.* **40**:1240). According to the Centers for Disease Control (CDC), mortality due to Alzheimer's disease in the United States increased 10-fold between 1979 and 1987 (0.4 per 100,000 to 4.2 per 100,000) (*See*, "Reported death rate for Alzheimer's is up tenfold since 1979," (1990) *Clin. Psychiatr. News* **18**:21).

Patients suffering from Alzheimer's disease typically suffer progressive memory deficit, progressive decline in cognitive functions, anxiety, depression, visuospatial and speech deficits, delusions, personality changes, motor skill deterioration, loss of verbal

ability, and incontinence. Eventually, patients are completely incapacitated and disoriented, requiring total care. The course of Alzheimer's disease ranges from less than three years to over 20 years before death occurs. However, in typical cases, it progresses at a fairly constant rate, with an average duration of 6 to 10 years.

Alzheimer's disease is not the only cause of dementia. Indeed, there are over fifty recognized causes of dementia. As some causes of dementia are amenable to treatment, differential diagnosis of patients suffering dementia is particularly important. Because at least 20% of clinically diagnosed patients were found at autopsy to have had conditions other than Alzheimer's disease, the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and Alzheimer's Disease and Related Disorders Association (ADRDA) refined the clinical diagnostic criteria for Alzheimer's disease (*See*, M.A. Jenike, "Psychiatry," *in Medicine*, Scientific American, New York, [1991], pages 15 (V):1-5). Based on these criteria, the diagnosis of Alzheimer's disease may be "definite" (*i.e.*, requiring examination of brain tissue), "probable" (*i.e.*, patients have deficits in two or more areas of cognition, insidious onset of disease, progressive worsening of memory and other cognitive functions, and normal consciousness levels), or "possible" (*i.e.*, patents meet the criteria for probable Alzheimer's disease, but exhibit variations in the disease course or have a systemic illness that is sufficient to cause dementia, but is not considered to be the cause of the dementia).

The numerous varieties of dementia, and variations in patient presentations, often make diagnosis problematic. Thus, the NINCDS/ADRDA criteria are very detailed, and necessitate the thorough examination of patients with suspected Alzheimer's disease. Currently, the only way to obtain a definite diagnosis is by post-mortem histological examination of brain tissue for the presence of senile plaques.

The histopathological lesions of Alzheimer's disease include neuritic or senile plaques, neurofibrillary degeneration, and granulovacuolar neuronal degeneration. The senile plaques usually contain a core of insoluble, amyloidic extracellular material ("β-amyloid) surrounded by a halo of neurofibrillary tangles and dystrophic neurons. The primary protein component of the amyloidic core of senile plaques is a 4.2 kd amyloid β peptide (often referred to as "Aβ"). A number of other proteins have also been identified

as components of senile plaques, including anti-chymotrypsin and apolipoprotein E. The major component of the neurofibrillary tangles is an abnormally phosphorylated microtubule-associated protein referred to as "tau." The β-amyloid protein in senile plaques is a small glycoprotein (*i.e.*, a 39-43 amino acid protein, derived from the larger amyloid precursor protein), that has been detected in non-neural tissues (*e.g.*, skin, subcutaneous tissue, and intestines) and blood vessels of Alzheimer's disease patients (*See*, Cutler, *supra*). Deposits of amyloid may be detected by their ability to bind specific dyes, such as Congo red or thioflavin S. Thus, it has been further hypothesized that detection of this protein may serve as a potential diagnostic aid in the assessment of patients suffering from dementia.

Alzheimer's disease usually arises spontaneously, although genetics play a role in development of the disease. For example, the histopathologic lesions of Alzheimer's disease have been regularly observed in the brains of older patients dying of Down's syndrome (*See*, Cutler, *supra*). It is known that the β-amyloid gene resides on chromosome 21. As Down's patients have an extra copy of this chromosome, there is an increased expression of this chromosome in these patients. It has been hypothesized that increased expression of the β-amyloid gene may regulate the formation of amyloid plaques ("senile plaques") in these patients. In addition, four loci were recently identified as playing a role in the genetic susceptibility of Alzheimer's disease (*See*, Pericak-Vance and Haines (1995) *Trends Genet.* **11**:504).

Other potential risk factors for the development of Alzheimer's disease include environmental factors (*e.g.*, head trauma, smoking, and exposure to heavy metals), sociological factors (*e.g.*, depression and educational level), biological factors (*e.g.*, increasing age and hyperthyroidism), and a family history of Alzheimer's disease, Down's syndrome, or Parkinson's disease (*See*, Pericak-Vance and Haines, *supra*). Nonetheless, despite recent advances, the exact etiology and pathogenesis of Alzheimer's disease remain largely unknown.

In addition to the problems associated with diagnosing Alzheimer's disease, improvements are needed in the area of treatment. Many agents have been tested for their ability to treat the cognitive decline associated with Alzheimer's disease. For example,

various cholinergic enhancers (*e.g.*, choline and lecithin) have been tested. Unfortunately, cholinergic precursors have been shown to be not useful, although some drugs that stimulate cholinergic transmission may be helpful in some patients. One example is physostigmine, a compound that prevents the synaptic breakdown of acetylcholine. However, the overall clinical effect of this drug has not been as dramatic as initially hoped (*See*, M.A. Jenike, "Psychiatry," *in Medicine*, Scientific American, New York, (1991), pages 13 (V):1-5).

Other drugs, such as tetrahydroaminoacridine (THA or tacrine), a centrally acting anti-cholinesterase, have been tested. In a large multi-center trial of THA, liver enzyme abnormalities were reported and the preliminary results indicated that, at least at low dosages, THA is not an effective treatment of Alzheimer's disease (Jenike, *supra*). Ergoloid mesylates (Hydergine) is an extremely safe compound, and remains the most commonly prescribed drug for patients with Alzheimer's disease. However, the overall effects of the drug are at best minimal.

The mechanisms of toxicity due to accumulation of β-peptide are currently the subject of much investigation, and no definitive causes of toxicity have yet been established. (See, Benzi and Moretti (1995) *Neurobiology of Aging*, **16**:661-674). Because of this uncertainty, efforts to develop mechanism-based treatment regimens have not been possible. Compounds presently in use to treat Alzheimer's disease only serve to alleviate the systemic effects associated with the disease.

In order to study disease mechanisms and genetic-based phenomena, animals in which a foreign gene has been inserted have been described by various researchers. International Patent Application WO 96/03034 describes insertion of retroviral vectors into fish, in order to produce fish with desirable traits or to study development. Various transgenic animal models for Alzheimer's disease are described in International Patent Applications WO 93/14200, WO 93/02189, WO 94/12627, WO 94/23049, and European Patent Publication EP 653154. Typically these transgenic animals are mice or other mammals; however, β-amyloid peptide has been expressed in the nematode *Caenorhabditis elegans* (Link (1995) *Proc. Natl. Acad. Sci.* **92**: 9368). The use of *C. elegans* for investigation of mutant forms of the *C. elegans* genes mec-4 and deg-1, which

cause neurodegeneration, is described in U.S. Patent No. 5,196,333. Drawbacks to mammalian animals as model systems are the relatively long generation time, which makes mammals less desirable for high-throughput screening of potential pharmaceuticals, and the difficulty in studying the molecular processes of interest without sacrificing the animal for cell and tissue analysis.

Reporter genes are genes that encode proteins or other compounds that can be detected by a variety of methods, and which "report" the occurrence of successful introduction and expression of gene sequences. β-galactosidase and luciferase are examples of such reporter genes. Recently, the gene for green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has been described in U.S. Patent No. 5,491,084. This gene can provide a method for indicating expression of a gene via fluorescent detection of GFP. The drawback to using GFP in mammalian systems is as described above, that is, the need to sacrifice the mammal in order to analyze the cells and tissues of interest. This drawback is especially severe in the context of large-scale screening of potential therapeutic compounds, and U.S. Patent No. 5,491,084 does not describe a system suitable for high-throughput screening of pharmaceuticals for activity against toxic proteins expressed in cells.

Thus, methods and animal systems are needed to screen drugs quickly and inexpensively for their effects on proteins and other substances associated with Alzheimer's disease. Convenient methods and animal systems for screening drugs for other neurodegenerative diseases, such as prion diseases, Huntington's disease, and amyotrophic lateral sclerosis, are also desirable.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions useful for the production and use of dually transgenic animals, in particular *Caenorhabditis elegans*. In addition, in one embodiment, the present invention provides transparent animals that express a reporter gene inducible by a toxic transgene. In a preferred embodiment, the toxic transgene encodes β-peptide. Although it is not intended that the present invention be limited to any particular reporter, in a preferred embodiment, the reporter gene is green fluorescence

protein (GFP). It is contemplated that the effects or product of the reporter gene be observable (*i.e.*, the reporter gene is expressed). For example, in embodiments in which GFP is the reporter, its presence is detected using fluorescence microscopy. In addition, in these embodiments, the animals do not need to be sacrificed in order to observe the expression of the reporter gene (*i.e.*, the detection may be accomplished on living animals). If other reporters are used, other detection methods may be necessary. For example, *lacZ* expression may be detected by exposing the tissues of the animal to the substrate for the gene (*i.e.*, β-galactoside), and observing for the presence of blue dye in the tissues. However, this method requires that the animal be sacrificed in order to observe the expression of the reporter gene.

In one alternate embodiment, the genome of the transparent animal further contains a heterologous marker gene. In a preferred embodiment, the marker gene is *rol-6(su-1006)*. In the embodiments in which *rol-6(su-1006)*, expression of the marker gene is accomplished by observing the living animals for the rolling behavior that results due to the expression of the abnormal collagen gene. In contrast, wild-type animals exhibit normal locomotion and do not exhibit this rolling behavior, as their collagen is normal.

In an alternative embodiment, the first transgene of the dually transgenic non-human animal comprises pCL25. In another alternative embodiment, the second transgene of the dually transgenic non-human animal comprises pCL12. In one alternate embodiment, the genome of the transparent animal further contains a heterologous marker gene. In a preferred embodiment, the marker gene is *rol-6(su-1006)*. In the embodiments in which *rol-6(su-1006)*, expression of the marker gene is accomplished by observing the living animals for the rolling behavior that results due to the expression of the abnormal collagen gene. In contrast, wild-type animals exhibit normal locomotion and do not exhibit this rolling behavior, as their collagen is normal.

In a preferred embodiment, the transparent animal is selected from the class Nematoda. Although it is not intended that the transparent animal of the present invention be limited to any specific animal, in a particularly preferred embodiment, the transparent animal is *Caenorhabditis elegans*.

In an alternate embodiment the cells of the transparent animal display toxicity resulting from the accumulation of β-peptide within the cells. In one preferred embodiment, the genome of the transparent animal comprises SEQ ID NO:5 and SEQ ID NO:8.

The present invention also provides methods for producing dually transgenic non-human animal comprising: providing: a first and second non-human animal; a first transgene comprising β-peptide; and a second transgene, comprising a reporter; introducing the first transgene into the genome of the first non-human animal to produce a first transgenic animal, and introducing the second transgene into the genome of the second non-human animal to produce a second transgenic animal; and mating the first transgenic animal with the second transgenic animal to produce a dually transgenic animal, wherein the β-peptide and the reporter are expressed.

In one embodiment of the methods of the present invention the dually transgenic non-human animal is transparent. In a preferred embodiment, the transparent animal is a nematode, while in a particularly preferred embodiment, the animal is *Caenorhabditis elegans*.

In an alternative embodiment, the first transgene of the dually transgenic non-human animal comprises pCL25. In another alternative embodiment, the second transgene of the dually transgenic non-human animal comprises pCL12. In one alternate embodiment, the genome of the transparent animal further contains a heterologous marker gene. In a preferred embodiment, the marker gene is *rol-6(su-1006)*. In the embodiments in which *rol-6(su-1006)*, expression of the marker gene is accomplished by observing the living animals for the rolling behavior that results due to the expression of the abnormal collagen gene. In contrast, wild-type animals exhibit normal locomotion and do not exhibit this rolling behavior, as their collagen is normal.

The present invention also comprises methods for testing compounds for anti-toxic effects, comprising: providing a dually transgenic non-human animal expressing a toxic transgene and a reporter; a composition comprising a test compound in a form suitable for administration such that the compound is bioavailable in the cells of the animal; and administering the test compound to the non-human animal. The toxic transgene induces

expression of the reporter gene. In a preferred embodiment, the toxic transgene is β-peptide and the reporter gene is GFP. In one embodiment, the method further comprises the step of measuring a reduction or increase in the expression of the reporter by the dually transgenic non-human animal and thereby identifying a compound as therapeutic. In a particularly preferred embodiment of the methods, the compounds inactivate the β-peptide expressed by the dually transgenic animal.

In one embodiment of the methods for testing compounds for β-peptide toxicity, the dually transgenic non-human animal is transparent. In a preferred embodiment, the transparent animal is a nematode, while in a particularly preferred embodiment, the animal is *Caenorhabditis elegans*. In an alternative embodiment, the first transgene of the dually transgenic non-human animal comprises pCL25. In another alternative embodiment, the second transgene of the dually transgenic non-human animal comprises pCL12. In one alternate embodiment, the genome of the transparent animal further contains a heterologous marker gene. In a preferred embodiment, the marker gene is *rol-6(su-1006)*, which produces effects described herein.

In one alternative embodiment, one or more test compounds are tested for their ability to counter the toxic effects of transgene product. It is also contemplated that the test compounds will be tested for their ability to prevent the expression of the toxic transgene, for example, β-peptide.

In alternative embodiments, dually transgenic animals in which the expression of, or effects of transgene toxicity, are reduced or eliminated by the test compounds, are mated. The progeny of these matings are also then tested for the effects of test compounds on the expression of, or effects of toxicity. In yet another embodiment, the progeny of these matings are used in other assay systems for the identification of therapeutic compounds.

In yet another alternative embodiment, the dually transgenic animals of the present invention are used to identify methods suitable for the diagnostic testing of Alzheimer's disease or pathology due to other diseases. Thus, the dually transgenic animals of the present invention are used to develop assays suitable for use in humans or animal models of Alzheimer's disease.

DESCRIPTION OF THE FIGURES

Figure 1 is a diagram illustrating the construction of dual transgenic animals expressing β-amyloid peptide and an hsp/GFP reporter.

Figure 2 shows the DNA sequence (SEQ ID NO:5) and restriction map for pCL12.

Figure 3 is a graphic map of pCL12.

Figure 4 shows the DNA (SEQ ID NO:6), and amino acid sequence (SEQ ID NO:7) of pCL12 from nucleotide 1071 through 1253 (*i.e.*, the β-(1-42) nucleic acid and amino acid sequence).

Figure 5 shows the DNA sequence (SEQ ID NO:8) and restriction map for pGFP-TT.

Figure 6 is a graphic map of pGFP-TT.

Figure 7 shows the DNA sequence (SEQ ID NO:9) and restriction map for pCL25.

Figure 8 is a graphic map of pCL25.

Figure 9 shows the DNA sequence (SEQ ID NO:10) of *rol*-6.

Figure 10 shows the amino acid sequence (SEQ ID NO:11) of *rol*-6.

DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions useful for the production and use of transgenic animals. The methods and transgenic animals of the invention also provide an efficient and effective system for screening drug effective in ameloriating the effects of toxic gene products.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. *See, e.g.*, Sambrook, Fritsch, and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, (F.M. Ausubel et al. eds., 1987); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); PCR 2: A PRACTICAL APPROACH (M.J. McPherson, B.D. Hames and G.R. Taylor eds., 1995); ANIMAL CELL CULTURE (R.I.

Freshney, Ed., 1987); and ANTIBODIES A LABORATORY MANUAL (Harlow et al. eds., 1987).

Animals

The methods and systems described herein can be practiced with any non-human animal. In a preferred embodiment, the animal used is transparent for at least part of its life cycle, for example, oocytes (*Xenopus* or others); larvae; pupae, fish (zebrafish or others); and nematodes, such as *C. elegans*. *C. elegans* is particularly preferred because it is transparent throughout its life and has been extensively studied as a model to determine developmentally regulated gene expression, as well as pattern formation. The visibility of all of the animals' cells throughout their life cycles, has led to the complete determination of the cell lineages, and a detailed description of the morphogenic changes that occur during *C. elegans* development. Rapid methods for introduction of cloned DNA into the *C. elegans* germ line has provided means to study molecular function and expression *in vivo*, as the effects of the gene expression are usually readily visible in the transparent worms.

Reporter Molecules

The methods and compositions of the present invention make use of reporter genes to monitor gene activity. Visible and quantifiable reporter genes are known and described in the art. Successfully used reporter molecules in gene fusion vectors in studies with *C. elegans* include the *E. coli lacZ* coding region (*See e.g.*, A. Fire *et al.*, (1990) *Gene* **93**:189-198), and the *Aequora victoria gfp* coding region (*See e.g.*, M. Chalfie *et al.*, (1994) *Science* **263**:802-805; D.C. Prasher *et al.*, (1992) *Gene* **111**:229-233; and Genbank Accession #M62654), which produces an intrinsically fluorescent protein. Although various fusion expression vectors have been used and reported in the literature, problems have been encountered (*See e.g.*, A. Fire, "Fire Lab Vector Kit"--June 1995). For example, with *lacZ*, there have been many reports in which it was not possible to correlate transgene expression patterns with physiological expectations, or only weak correlations have been possible. Ectopic expression is often frequently seen with short promoters, and occurs

most prominently in the gut and pharynx of the affected animals. This may be due to weak promoter and/or enhancer signals in the vectors. Expression pattern deficits of *lac*Z fusions have been classified into three groups. In the first, transgenes are expressed in the correct tissue, but mosaic expression is observed (*i.e.*, only a subset of the cells stain during the detection methods). This has been observed even with integrated high-copy transgenes. In the second group, expression in a single tissue or cell population is not seen with the transgene. Finally, there has been the failure of transgene fusion constructs to show expression in the pre-12 cell embryo, or in any embryonic, larval, or adult germ line.

The *gfp* fusion vectors were developed as an alternative to the *lac*Z markers, but have been available for a much shorter time (*See e.g.*, Chalfie *et al.*, (1994), *supra*; U.S. Patent No. 5,491,084). Initial reports indicate that the fluorescence pattern appears to be more restricted than that exhibited by equivalent *lac*Z fusion. In addition, there are still problems associated with the germline expression of the transgenes and ectopic expression. Indeed, in some cases, the problems with ectopic expression have been exacerbated by these "improvements."

The present invention overcomes many of these problems described in the art. The tranparent animals and dually transgenic animals described herein provide easily visible, stably expressed systems in which the toxic transgene and reporter gene are expressed similarly to endogenous chromosomal gene expression. These animals provide an excellent system for screening compounds having effects on the toxic transgene. The present invention, therefore, provides model systems for the study of human diseases and methods of identifying therapeutic compounds using these animal systems.

Inducible Promoters

As discussed above, the reporter genes are operably linked to an inducible promoter. The promoter is induced by the toxic transgene, for example by the gene product of the toxic transgene. It is also contemplated that the toxic transgene can act to induce the promoter indirectly, for example by disrupting other cellular proteins or functions. Suitable inducible promoters are available and can be readily determined by those skilled in the art. Non-limiting examples of promoters which are induced by

"stresses" include the metallothionein gene promoter (*mtl*-1 or *mtl*-2; *e.g.*, Genbank Accession #M92910, #M11794, #X00504, and #X00953), and the *C. elegans* amyloid precursor protein (APP) homolog *apl*-1 gene promoter (*See e.g.*, Daigle and Li, Proc. Natl. Acad., Sci., 90:12045-12049 [1993]). Other potential promoters include those from other inducible heat-shock genes; at least one of the known *C. elegans hsp*70 genes is strongly heat-inducible. It is also contemplated that promoters from genes known to be up-regulated under stress conditions in other systems (*e.g.*, superoxide dismutase, catalase, glutathione reductase, etc.) may also be useful.

In one embodiment, as described in the Examples below, a strong muscle-specific promoter was used to express a potentially secretable form of the β-peptide, so as to generate significant extracellular levels of β-peptide. This was accomplished in order to mimic the situation that may exist in the human brain, and allow observations of cell-external neurotoxicity. The present inventor has also demonstrated that the promoter must be chosen so that enough β-peptide is produced to cause physiological effects, but not so much to kill the animals.

The *unc*-54/β-(1-42) minigene was constructed with a modified signal sequence that has been previously shown to allow secretion of a *her*-1 protein product that is ectopically expressed in muscle cells (M.D. Perry *et al.*, (1993) *Genes Dev.*, 7:216-228). The development of *C. elegans* transgenic for expression of β-amyloid was previously described by the inventor (Link, (1995) *Proc. Natl. Acad. Sci.*, **92**:9368-9572).

However, in early experiments, it was observed that β-peptide deposits were not convincingly detected outside of the muscle cells, when tested with the antibodies described in Example 4, below. While an understanding of the mechanism is not necessary for the practice of the present invention, it is apparent that the majority of β-peptide expressed by the transgenic worms is retained in the muscle cells and is responsible for the pathology observed in the muscle cells.

It is also contemplated that β-peptide expression will be directed to other tissues, through utilization of appropriate promoters. For example, it is contemplated that animals expressing β-peptide in the intestine may be particularly useful to analyze compounds such

as drugs for their effect on β-peptide, as these cells readily take up exogenous compounds administered orally.

Transgenes

The transgene element of the present invention can be any sequence which is able to induce the promoter operably linked to the reporter gene. As noted, the transgene is "toxic" in the sense that it disrupts cellular function in some way. Preferably, the transgene encodes a protein that is toxic to the host cells and/or organism in that it causes deleterious effects to the host, for instance interfering with the hosts ability to survive and/or grow. The toxic transgenes encode proteins that disrupt cellular function directly (*e.g.*, the gene product is toxic) or indirectly (*e.g.*, the sequence of the transgene disrupts cellular function by some mechanism other than its gene product). Toxic proteins are distinguishable from simple chemical toxins (*e.g.*, heavy metals and the like) by their antigenicity and higher molecular weight.

In one embodiment, the toxic transgene comprises a gene encoding for an amyloidic protein, for example beta amyloid peptide, prion protein variants, transthyretin variants, gelsolin variants, cystatin variants, lysozyme variants and the like. In another embodiment, the transgene encodes a protein containing polyglutamine resulting from triplet-repeat expansion such as huntingtin (a protein that has been implicated in Huntington's Chorea), ataxin-1 or ataxin-2. Alternatively, proteins associated with inherent amyelotopic lateral sclerosis (ALS) for example, superoxide dismutase 1 variants and over-expressed neurofilament protein, can be used. It will be understood that the transgene can encode for an entire toxic protein or, alternatively, a functional (*i.e.* toxic) fragment.

Transgenic Animals

In one aspect, the present invention includes a dually transgenic non-human animal. In one embodiment, the development of these dually transgenic animals involves the production of two *C. elegans* lines with a single transgene in each line, designated as CL2005 and CL2070. *C. elegans* was chosen due to the observation that the toxic effect of

β-amyloid has been reported to occur in these animals, in addition to humans. Line CL2005 exhibited muscle-specific expression of human β peptide, while line CL2070 exhibited stress-inducible expression of GFP. GFP was incorporated into the present invention as it provides a marker (*i.e.*, "reporter") that is readily visible in living worms. Thus, dually transgenic animals both express β-peptide in their muscle cells and under appropriate conditions for observation (*i.e.*, fluorescence microscopy), exhibit green fluorescence in these cells.

Mating of these lines resulted in the production of dually transgenic animals, in which the presence of β-amyloid was easy to detect, due to the fact that the dually transgenic animals expressing β-amyloid glowed green. Thus, the present invention provides an easily detectable method for the specific expression of β-peptide in dually transgenic animals. This ease of detection provides great advantages for the development of methods to analyze the effects of β-amyloid *in vivo*. In addition, because the dually transgenic animals are not killed in order to detect the presence of β-amyloid, as would be required if other reporters (*e.g.*, *lac*Z or luciferase) were used, the animals may be useful for screening compounds for their effects on β-amyloid in an *in vivo* situation. Thus, the use of an reporter which is detectable *in vivo* provides significant advantages over currently available methods.

Other Advantages of Transgenic *C. elegans*

The present invention also includes methods of screening compounds for their ability to prevent or inhibit toxicity due to the toxic transgene. In these drug screening embodiments, the methods and animals described above can be used to identify potential therapies. For example, in assays to develop compounds to block the expression of β-amyloid or counter its toxic effects, transgenic worms (*e.g.*, green fluorescing worms with muscle-specific β-peptide expression), would be observed for their loss of fluorescence. In assays where the compound is effective in countering the expression of β-peptide or effects of β-peptide, the animals would no longer glow green. It is easy to visually screen and quantify (using commercially available equipment) for animals that have lost the ability to glow, and then use them to detail the interaction between the compound and the toxic

transgene. The present invention provides a fast and efficient screening system, for example by using commercially available equipment for assaying multiple compounds at once.

Thus, the transgenic animals of the present invention expressing a detectable reporter gene triggered by a toxic transgene provide animal models for human diseases. For instance, a reporter gene (such as GFP) induced by expression of β-peptide provides an animal model for human Alzheimer's disease. It is also not intended that the present invention be limited to animals expressing β-peptide in conjunction with GFP. It is contemplated that expression of other proteins associated with dementia and/or Alzheimer's or other diseases would also find use in conjunction with GFP in *C. elegans* as well. For example, it is contemplated that proteins (including mutated versions of proteins) such as the presenilins associated with Alzheimer's disease (*See e.g.*, J. Marx (1996) *Science* **274**:1838-1840) will be used in conjunction with GFP and full-length APP in dually transgenic *C. elegans*. Other toxic proteins which provide animal models for other human diseases are described herein.

These transgenic animals also provide a means to screen compounds for their ability to decrease or eliminate the toxicity. Examples of compounds suitable for testing using the transgenic animals of the present invention include, but are not limited to, Congo Red, tumor necrosis factor (TNF), estrogen, tacrine (9-amino-1,2,3,4-tetrahydroacridine), dihydroepiandrosterone (DHEA), compounds that inhibit ApoE4, and others, commercially available from suppliers such as Sigma. Compounds to be tested for anti-toxic activity are administered to the same number of dually transgenic animals (*e.g.*, generated using the methods described in the Examples, below) from the control group and the treatment group, and the presence or absence of reporter gene used as a measure of efficacy.

The compounds being tested can be administered using any suitable route (*e.g.*, oral, parenteral, controlled-release transdermal methods, and implants, etc.). In one preferred route, the compounds to be tested are suspended in the growth media provided to the worms. Generally speaking, the route of administration will depend on the stability of the compound, the susceptibility of the compound to "first pass" metabolism, the

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concentration needed to achieve a therapeutic effect, and the like. Following initial screening, a compound that appears promising (*i.e.*, which increases the number of worms which display reduced β-peptide toxicity) is further evaluated by administering various concentrations of the compound to additional transgenic animals in order to determine an approximate therapeutic dosing range.

Another screening method involves the crossing of the transgenic worms of the present invention with other transgenic worms. The animals are observed after treatment, in the presence and absence of the test compound(s), with the effects on the toxic transgene being gauged either by crude survival or the presence/absence of the reporter gene. It is also contemplated that the methods of the present invention be modified so as to provide means to analyze disease-related proteins believed to have dominant toxic effects, by substituting appropriate sequences for the β-peptide sequences used as described in the Examples. For example, proteins such as transthyretin (known to be associated with familial amyloid polyneuropathy; *See e.g.*, Christmason *et al.*, (1991) *FEBS* **281**:177-180; Genbank Accession #D00096), and variant superoxide dismutase (known to be associated with familial amyotrophic lateral sclerosis [Lou Gehrig's disease]), prion proteins, A4 amyloid protein (*See e.g.*, Ponte *et al.*, (1988) *Nature* **331**:525-527; Salbaum *et al.*, U.S. Patent No. 5,151,508, herein incorporated by reference), APP (*See e.g.*, Kitaguchi *et al.*, (1988) *Nature* **331**:530-532; Sata *et al.*, EP Appln. 94117512.7; Scott *et al.*, WO 9412627; Wadsworth *et al.*, WO 9314200; Gearhart *et al.*, WO 9423049; and Neve *et al.*, WO 9302189, all of which are herein incorporated by reference), other amyloidic proteins (*e.g.*, variant lysozymes and amylin peptide), and other proteins associated with neuronal degeneration (*See e.g.*, U.S. Patent No. 5,196,333), will be used in the methods of the present invention by substituting the appropriate nucleic acid sequences encoding the protein of interest for the β-peptide described in Example 1. It is also contemplated that animals transgenic for dual proteins (*e.g.*, β-peptide in combination with another protein, such as transthyretin) will be used. Thus, the present invention provides the means to analyze the effects of numerous genes and proteins *in vivo*.

Furthermore, the transgenic animals of the present invention provide distinct advantages over other transgenic animals currently used to analyze diseases such as

Alzheimer's disease. Due to the short gestation period of *C. elegans*, transgenic animals can be produced much more rapidly than when mammals, such as mice are used. For example, transgenic mice overexpressing a 695 amino acid isoform of β-amyloid precursor did not show learning and memory impairment until they were 9-10 months of age (Hsiao *et al.*, Science 274:99-102 [1996]). In contrast, the transgenic animals of the present invention express β-peptide and may be manipulated at a very early age, even in embryonic stages.

In addition, because the transgenic animals are easy to select based on the presence of the green fluorescence, invasive procedures such as surgery, necessary to analyze the effects of the toxic transgene in other animals (*e.g.*, mice) are avoided.

Finally, the apparent toxicity of β-peptide in transgenic animals was found to be temperature-dependent. Transgenic animals maintained at 25.5°C were significantly more sick than those maintained at 16°C. The animals maintained at the higher temperature became paralyzed more quickly, failed to eat or grow, failed to show normal egg-laying, and many died before reaching adulthood. However, wild-type animals grow well at either of these temperatures. The CL2005 parental line was temperature-sensitive for viability, as it was not possible to propagate this line at the elevated temperature. In the CL2070 parental line, 25.5°C maintenance was not sufficient to induce the hsp/GFP construct. The hsp/GFP response was similarly temperature-dependent. It was difficult to detect GFP when the dual transgenic animals were raised at 16°C, but GFP induction was dramatic when the animals were raised at 25.5°C.

These temperature dependency observations were exploited by the propagation of animals at 16°C, and then upshifting them to 25.5°C, when GFP induction was desired. This effect also has potential benefits in the analysis of compounds, as the animals can be pre-incubated in the presence of drugs or other compounds at the lower temperature before the upshift to the higher temperature, in order to ensure that the presumed protective effect of the drug was in place, prior to the strong induction of β-peptide toxicity. Importantly, these assays may be completed within one day and are suitable for rapid methods (*e.g.*, the use of a microtiter format and a plate fluorimeter), so that literally thousands of compounds may be tested simultaneously.

In sum, the present invention provides methods and compositions useful as animal models for disease, as well as providing methods and compositions for disease therapy and prevention. The animal testing may be supplemented and confirmed by testing on human subjects. However, the transgenic animals of the present invention allow the testing of a large number of compounds, both various methods, including those known in the art.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The term "non-human animal" includes vertebrates such as rodents, arthropods, insects (*e.g.*, Diptera), fish (*e.g.*, zebrafish), non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc. The non-human animal can be used at any stage in its development, for example oocyte, fetal, larval, pupal stages or the like. Preferred non-human animals are those that are transparent, such as certain nematodes, oocytes, larvae and fish.

As used herein, the term "transparent" is used in references to animals through which light will be transmitted. However, it is not intended that the amount of light transmittance be limited to any particular amount. For example, an animal is transparent so long as at least some light may be transmited through its body. Non-limiting examples of transparent animals are larval stages of some animals (*e.g.*, flies) and oocytes (*e.g.*, *Xenopus* oocytes). Particularly preferred non-human animals are selected from the nematodes (*i.e.*, any animal in the Class Nematoda), most preferably *Caenorhabditis elegans*. However, it is contemplated that other transparent animals, such as zebrafish will be useful in the present invention. For example, transgenic zebrafish have been produced (*See e.g.*, Lin *et al.*, PCT Publ. WO9603034; incorporated herein by reference).

The "non-human animals having a genetically engineered genotype" of the invention are preferably produced by experimental manipulation of the genome of the germline of the non-human animal. These genetically engineered non-human animals may be produced by several methods including the introduction of a "transgene" comprising nucleic acid (usually DNA) into an embryonal target cell or integration into a chromosome of the somatic and/or germ line cells of a non-human animal by way of human

intervention, such as by the methods described herein. The process by which a DNA molecule becomes stably incorporated into another genome is referred to as "stable integration." Non-human animals which contain a transgene are referred to as "transgenic non-human animals". A transgenic animal is an animal whose genome has been altered by the introduction of one or more transgenes.

The term "transgene" as used herein refers to a foreign gene that is placed into an organism by introducing the foreign gene into gonadal cells, embryonic cells, newly fertilized eggs, or early embryos. The term "foreign gene" refers to any nucleic acid (*e.g.*, gene sequence) which is introduced into the genome of an animal by experimental manipulations and may include gene sequences found in that animal so long as the introduced gene contains some modification relative to the naturally-occurring gene. A preferred foreign gene is the β-amyloid gene (*e.g.*, β-peptide), or fragments thereof. A "toxic transgene" refers to a foreign gene which disrupts cellular function in some way. For example, the toxic transgene may produce a gene product (*i.e.*, protein) which is toxic to the cell or organism. Alternatively, the transgene may disrupt other cellular proteins, or act as a DNA binding site without producing a protein.

As used herein, the term "toxicity" refers to the production of toxic effects by any compound or substance. For example, while it is not necessary to the understanding and use of the present invention, β-peptide toxicity may arise due to the accumulation of β-peptide molecules. There are numerous manifestations of toxicity that may occur. For example, the toxicity associated with β-peptide may be manifested as negative effects on muscle cells of dually transgenic animals.

The terms "promoter element, " "promoter," or "promoter sequence" as used herein, refer to a DNA sequence that is located at the 5' end of (*i.e.*, precedes) a gene in a DNA polymer and provides a site for initiation of the transcription of the gene into mRNA. An "inducible promoter" is a promoter that is triggered by certain signals within the cell, for instance binding of a transcription factor, stress, heat or the like.

The term "reporter gene" as used herein refers to genes that encode proteins or other compounds that can be detected by a variety of methods. These genes "report" the occurrence of successful introduction and expression of sequences such as transgenes.

Non-limiting examples of reporter genes include antibiotic resistance genes, genes encoding enzymes and genes encoding other detectable proteins. Expression of the reporter gene is detected using methods known in the art. In a preferred embodiment, the reporter gene is a GFP gene, although *lac*Z β-galactosidase gene, or any other reporter system may be used to detect the successful production of transgenic animals. In particularly preferred embodiments, the reporter is a compound or protein which may be present or expressed within living animals. That is, it is not necessary to sacrifice the animal in order to detect the presence of the reporter. The type of the reporter gene system used is not critical to the invention, and it is contemplated that any system suitable for use with the transgenic animals of the present invention will be used.

As used herein, the terms "dual trangenic" and "dually transgenic" refer to animals or cells in which more than one transgene have been introduced. For example, the term is used in reference to cells which contain the sequences encoding β-peptide, and sequences encoding a reporter (*e.g.*, GFP). However, it is not intended that the number of transgenes in the dually transgenic animals of the present invention be limited to two. For example, the transgenic animals may also contain another one or more marker genes (*e.g.*, *rol-6* sequences), in addition to β-peptide and reporter sequences. In the embodiments in which *rol-6(su-1006)*, expression of the marker gene is accomplished by observing the living animals for the rolling behavior that results due to the expression of the abnormal collagen gene. In contrast, wild-type animals exhibit normal locomotion and do not exhibit this rolling behavior, as their collagen is normal.

The transgenic animals of the present invention are preferentially generated by introduction of the targeting vectors into gonad cells. Transgenes can be efficiently introduced into the cells by DNA transfection using a variety of methods known to the art, including electroporation, calcium phosphate co-precipitation, protoplast or spheroplast fusion, lipofection, and DEAE-dextran-mediated transfection. Transgenes may also be introduced into cells by retrovirus-mediated transduction or by micro-injection. In one preferred embodiment, the transgenes are injected into gonads of *C. elegans* as described by Mello *et al.* (1991) *EMBO J.*, **10**:3959-3970. Alternative methods for the generation of transgenic animals containing an altered gene are known to the art. For example,

embryonal cells at various developmental stages can be used to introduce transgenes for the production of transgenic animals. Different methods are used depending on the stage of development of the embryonal cell.

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting related sequences include oligolabeling, nick translation, and end-labeling or PCR amplification using a labeled nucleotide. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. The term may also be used in reference to proteins. For example, a variety of protocols which employ polyclonal or monoclonal antibodies specific for the β-peptide protein product are known in the art (*See*, the Examples). These antibodies can be used as markers for the expression of proteins. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS).

The term "gene" refers to a DNA sequence that comprises coding sequences necessary for the production of a polypeptide or precursor. In some instances, a gene can also include control sequences. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired enzymatic activity is retained.

The term "gene of interest" refers to any gene, the manipulation of which may be deemed desirable for any reason, by one of ordinary skill in the art.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are

tified by the fact that they have altered characteristics when compared to the wild-type or gene product.

The terms "targeting vector" or "targeting construct" refer to oligonucleotide nces comprising a gene of interest flanked on either side by regulatory sequences. ably, the targeting vector is capable of homologous recombination such that the gene rest is integrated by recombination.

As used herein, the terms "vector" and "vehicle" are used interchangeably in ce to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The terms "expression vector" or "expression cassette" as used herein, refers to a inant DNA molecule containing a desired coding sequence and appropriate nucleic uences necessary for the expression of the operably linked coding sequence in a r host organism. Nucleic acid sequences necessary for expression in prokaryotes nclude a promoter, an operator (optional), and a ribosome binding site, often along r sequences. Eukaryotic cells are known to utilize promoters, enhancers, and n and polyadenylation signals.

e terms "in operable combination," "in operable order," and "operably linked" as n, refer to the linkage of nucleic acid sequences in such a manner that a nucleic ule capable of directing the transcription of a given gene and/or the synthesis of rotein molecule is produced. The term also refers to the linkage of amino acid n such a manner so that a functional protein is produced.

term "tissue-type specific" as it applies to a promoter, refers to a promoter that f selectively directing expression of a gene in a specific tissue. Similarly, the ue-specific promoter in the method of the present invention does not require cificity. In general, the requisite specificity is found where a plurality (or, bly, a majority) of cells in one tissue type express a gene of interest, while .g., greater than 80%, and preferably greater than 90%, and more preferably 5%), of the cells in other tissue types do not. In one embodiment of the ion, the strong muscle promoter *unc*54 was used (*See*, Example 1).

d herein the term "portion" when in reference to a gene refers to fragments of fragments may range in size from a few nucleotides to the entire gene

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sequence minus one nucleotide. Thus, "an oligonucleotide comprising at least a portion of a gene" may comprise fragments of the gene or the entire gene.

As used herein, the term "substantially purified" refers to molecules, either nucleic or amino acid sequences, that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated. An "isolated polynucleotide" is therefore a substantially purified polynucleotide.

As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis disclosed in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,965,188, all of which are hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization

with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; and/or incorporation of ^{32}P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications. Amplified target sequences may be used to obtain segments of DNA (*e.g.*, genes) for the construction of targeting vectors, transgenes, etc.

As used herein, the terms "PCR product" and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

The term "Central Nervous System" refers to the "spinal cord" and the "brain." The spinal cord comprises white areas and grey areas. The grey area contains nerve cell bodies, whereas the white area is essentially comprised of myelinated nerves. The brain, which is also known as the encephalon, is that portion of the cerebrospinal axis which is contained in the cavity of the cranium. The brain comprises the two cerebral hemispheres, the inter-brain, the mid-brain, the pons Varolli and cerebellum, and the medulla oblongata. The two hemispheres together with the parts derived from the thalamencephalon form the forebrain. The two cerebral hemispheres are separated by the longitudinal fissure and also comprise the bylvian fissure, the fissure of Rolando, and the parieto-occipital fissure. The lobes on the external surface of the brain comprise the frontal lobe, the parietal lobe, the occipital lobe, and the temporal lobe. Placed along the middle line of the brain are, among others, the rostrum and peduncles of corpus callosum, lamina cinera, optic commissure and the pituitary body. On each side of the middle line lies the frontal lobe, olfactory lobe and the hemisphere of cerebellum.

The terms "neuron," "neural cell," and "nerve cell" are used interchangeably to refer to a cell which is located in the nervous system. Nerve cells are composed of the nerve cell body (perikaryon), one or more dendrites, and an axon. Neurons can be classified according to the number of processes originating from the cell body. Thus,

unipolar neurons have a single process, bipolar neurons have one axon and one dendrite, while multipolar neurons (which are the most common) comprise more than two processes. The term "neuron" comprises cholinergic neurons and sensory neurons. As used herein, the term "cholinergic neuron" means a neuron in the Central Nervous System (CNS) and in the Peripheral Nervous System (PNS) whose neurotransmitter is acetylcholine. As used herein, the term "sensory neuron" includes a neuron which is responsive to environmental cues (*e.g.*, temperature and movement) from, for example, the skin, muscle and joints of a mammal.

The term "nerve" refers to two or more neurons arranged in linear sequence such that the axon of one neuron establishes a structural and functional link with the dendrite of a second neuron to form a "synapse."

The term "compound" refers to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function. Compounds comprise both known and potential therapeutic compounds. A compound can be determined to be therapeutic by testing using the testing methods of the present invention (*i.e.*, a "test compound"). A "known therapeutic compound" refers to a therapeutic compound that has been shown (*e.g.*, through animal trials or prior experience with administration to humans) to be effective in such treatment or prevention. In other words, a known therapeutic compound is not limited to a compound efficacious in the treatment or prevention of neural related disorders.

A compound is said to be "in a form suitable for administration such that the compound is bioavailable in the blood of the animal" when the compound may be administered to an animal by any desired route (*e.g.*, oral, intravenous, subcutaneous, intramuscular, etc.) and the compound or its active metabolites appears in the blood of the animal in an active form. Administration of a compound to a pregnant animal may result in delivery of bioavailable compound to the embryonic progeny of the animal.

The "wild-type β-amyloid" or "β-peptide" gene and gene product refers to the nucleotide and amino acid sequences provided in SEQ ID NOS:6 and 7, respectively. Those skilled in the art will be well aware that certain modifications of SEQ ID NOS:6 and 7 can be made which will not interfere with the production of a polypeptide having an

activity indistinguishable from that of the wild-type β-amyloid; the present invention specifically contemplates these variant forms of β-amyloid. A "variant" of the β-peptide is defined as an amino acid sequence that differs by one or more amino acids. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (*e.g.*, replacement of leucine with isoleucine). More rarely, a variant may have "nonconservative" changes, *e.g.*, replacement of a glycine with a tryptophan. Similar minor variations may also include amino acid deletions or insertions (*i.e.*, additions), or both. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNAStar software.

The term "an oligonucleotide sequence comprising at least a portion of a human β-amyloid gene" refers to a polynucleotide sequence (*i.e.*, a nucleic acid sequence) containing a nucleotide sequence derived from a human β-amyloid gene. This sequence may encode a portion or all of the β-amyloid protein; alternatively, this sequence may contain non-coding regions derived from the β-amyloid gene or a combination of coding and non-coding regions. The oligonucleotide may be RNA or DNA and may be of genomic or synthetic origin.

As used herein the term "portion" when in reference to a gene refers to fragments of that gene. The fragments may range in size from 10 nucleotides to the entire gene sequence minus one nucleotide. Thus, "an oligonucleotide comprising at least a portion of a gene" may comprise fragments of the gene or the entire gene.

An animal whose genome comprises a "heterologous marker gene" is an animal whose genome contains a marker gene not naturally found in the animal's genome. In one preferred embodiment of the present invention, the heterologous marker gene is a mutant collagen gene, such as the *rol-6(su-1006)* gene. However, it is intended that other marker genes will be used with success in the present invention, including other mutant collagen genes, as well as other marker genes commonly known to those in the art.

As used herein, the term "diagnostic assay" refers to methods for the diagnosis of disease, illness, and/or pathology. It is intended that the term encompass any methods for

diagnosis, including, but not limited to assays based on immunoreactivity (*e.g.*, radioimmunoassays, fluorescence immunoassays, enzyme immunoassays), histochemistry, dye retention or binding (*e.g.*, fixing of dyes such as Congo Red), nucleic acid based diagnostic methods (*e.g.*, identification of nucleic acid sequences associated with disease or pathology), etc.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); μM (micromolar); N (Normal); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); μg (micrograms); ng (nanograms); l or L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nm (nanometers); °C (degrees Centigrade); U (units), mU (milliunits); min. (minutes); sec. (seconds); % (percent); kb (kilobase); bp (base pair); PCR (polymerase chain reaction); β-(1-42) amino acids 1-42 of β-amyloid peptide; Tris (tris(hydroxymethyl)-aminomethane); BSA (bovine serum albumin); Fisher (Fisher Scientific, Pittsburgh, PA); Sigma (Sigma Chemical Co., St. Louis, MO.); Promega (Promega, Corp., Madison, WI); Perkin-Elmer (Perkin-Elmer/Applied Biosystems, Foster City, CA); Senetek (Senetek, PLC, Maryland Heights, MO); Boehringer Mannheim (Boehringer Mannheim, Corp., Indianapolis, IN); Stratagene (Stratagene, Inc., La Jolla, CA); and NEB (New England Biolabs, Beverly, MA). Unless otherwise indicated, the restriction enzymes used in these Examples were obtained from NEB. *C. elegans* is available from the Caenorhabditis Genetics Center, at the University of Minnesota, St. Paul, MN.

EXAMPLE 1

Assembly of the β-(1-42) Minigene

1-42) minigene used in subsequent experiments (*See*,
ic *C. elegans* engineered to express amyloidic human
scribed below, these animals contained constructs in
4 promoter/enhancer of *C. elegans* drove the expression of
lerived from human β-amyloid cDNA clones, as described
man β-amyloid peptide in transgenic *Caenorhabditis*
, 92:9368-9372 [1995]).

ng the 42 amino acid β-amyloid peptide derived from
n cDNA was assembled. The artificial signal peptide
52.81 was amplified under standard conditions, using
'GGCCAAAGGACCC-3')(SEQ ID NO:1), and "SP-down"
CCAGCAAGAT-3')(SEQ ID NO: 2), cleaved with *Nhe*I
id inserted between the unique *Nhe*I and *Kpn*I sites of
construct "pCL2." This process resulted in a re-
e, such that the signal peptide cleavage site, as predicted by
von Heijne, Nucl. Acids Res., 14:4683-4690 [1986]),
Gly-Thr dipeptide encoded by the *Kpn*I site.

ding amino acids 1-42 of β-amyloid, and which contained
olified using standard methods, from human β-amyloid
4T4B (P. Ponte *et al.*, Nature 331:525-527 [1988]), by
GGGGTACCGATGCAGAATTCCGACATGA-3') (SEQ
5'-CCCGAGCTCACGCTATGACAACACCGCCAA-
cation product was cleaved with *Kpn*I and *Sac*I, and
I and *Sac* I sites of pCL2, to generate "pCL3."
42) minigene fragment was removed from this plasmid by
d inserted between the unique *Nhe*I and *Sac*I sites of
The sequence of the β-(1-42) minigene was confirmed by
coding strand only, by techniques known in the art. The
):5), is shown in Figure 2. A graphic map of pCL12 is
ows the DNA and amino acid sequences (SEQ ID NOS:

6 and 7, respectively) of pCL12 from nucleotide 1071 through 1253 (*i.e.*, the β(1-42) nucleic acid and amino acid sequences) present in the construct.

EXAMPLE 2

Construction of the hsp/GFP Reporter

ımple, the expression vector ("pCL25") containing the hsp/GFP reporter
:xpression of the GFP-TT gene was constructed. This vector was used in
ments (*See*, Example 3) to produce transgenic *C. elegans* was
'-TT is a modified form of GFP that contains Ser65Thr and Ile167Thr
relative to the sequence of the wild-type GFP). The substitutions present
ce a protein which results in the production of a much brighter
ı the wild-type GFP.

ıs constructed as described below. A 431 bp *Hin*dIII-*Bam*HI fragment
p16-2 promoter was excised from the plasmid "pPD49.78," by digestion
*Hin*dIII. This 431 bp *Hin*dIII-*Bam*HI fragment was inserted between the
de #1) and *Bam*HI (nucleotide #31) sites of the GFP-TT gene in the
TT," (available from Yishi Jin, at the University of California, Santa
igase (Promega). The pGFP-TT plasmid contains the coding regions for
ı into a Tu61 backbone. The DNA sequence of pGFP-TT (SEQ ID NO: 8)
re 5. A schematic map of pGFP-TT is shown in Figure 6, in which unique
ıre shown. The pGFP-TT plasmid was digested with *Hin*dIII and *Bam*HI,
ragment containing the hsp16-2 promoter was inserted, in order to
ss-inducible expression vector "pCL25." The DNA sequence of pCL25
and restriction map are shown in Figure 7. Figure 8 provides a schematic
ı which unique restriction sites are shown.

EXAMPLE 3

Construction of Transgenic Animals

In this Example, the transgenic parent animals were produced, with one line expressing the β(1-42) minigene (designated as "CL2005") and the other line expressing the hsp/GFP reporter (designated as "CL2070"). For both lines, the transgenes were introduced into *C. elegans* by gonad microinjection as known in the art and described by Mello *et al.* (Mello *et* EMBO J., 10:3959-3970 [1991]). Marker plasmid pRF4 containing the gene (*rol-6*[su1006]) (SEQ ID NO:9)(pRF4 carries a 4 kb *Eco*RI fragment of *C. elegans* genomic DNA containing the *rol*-6[su1006] collagen gene in the Bluescribe vector [Stratagene]: *See*, Mello *et al.*, *supra*; and Kramer *et al.*, Mol. Cell. Biol., 10:2081-2090 [1990]) was coinjected with the constructs, at approximately 100 ng/μl for each plasmid, into morphologically wild-type animals, and Roller transgenic progeny were recovered. The (*rol-6*[su1006]) gene (SEQ ID NO:9), contained within the pRF4 plasmid is a mutated *C. elegans* collagen gene, the expression of which produces the dominant, distinctive "Roller" phenotype. Figures 9 and 10 show the DNA and amino acid sequences of the *rol*-6 gene used in this Example, respectively.

Transmitting lines were established and maintained by selection for the Roller marker phenotype. Transgenic animals produced in this manner maintain the injected DNA as an extrachromosomal, multicopy array of variable mitotic and meiotic stability. Strains containing chromosomally integrated transgenes were recovered by irradiation of lines containing extrachromosomal transgenic arrays with 7000 rad (1 rad = 0.01 Gy) of gamma rays from a Cesium-66 source. Progeny of irradiated animals were then screen for 100% transmittance of the marker transgene. The transgenes in both the CL2005 and CL2070 lines were chromosomally integrated, and were 100% stable.

Transgenic (*i.e.*, as indicated by the Roller phenotype) animals produce both transgenic (*i.e.*, Roller) and non-transgenic (*i.e.*, non-Roller) progeny. These non-transgenic progeny were found to serve as good internal controls for phenotypic and immunohistochemical comparisons. The expression of GFP in the dual transgenic animals can be detected in less than 24 hours after the upshift of animals from 16 to 25.5°C, at all

stages of development, from late embryonic to adult. Expression of GFP resulted in the production of green fluorescence in muscle cells; intense tissue-specific expression of GFP was observed. The fluorescence can be observed using compound or dissecting epifluoroescence microscopy (*i.e.*, with standard fluorescein excitation and emission filters). It is also contemplated that the fluorescence is observable by use of fluorimeters and cell sorters.

EXAMPLE 4

Immunohistochemistry of Transgenic Animals

In this Example, immunohistochemistry was used to confirm the transmittance of the chimeric constructs in large populations of putative integrated lines.

As described by Link (Link, 1995, *supra*), whole mount specimens were prepared by fixing animals in 4% paraformaldehyde and permeabilizing them with 2-mercaptoethanol collagenase as known in the art and described by Link *et al.* (Link *et al.*, Genetics 131:867-881 [1992]). Three antibodies were used (polyclonal rabbit anti-β peptide antibody, Boehringer Mannheim; mouse monoclonal antibody Ab 4G8, available from Senetek; and monoclonal 4.1, a gift from B. Cordell at Scios Nova). The monoclonal 4.1 recognizes residues 8-15 of β-peptide.

As was observed by Link (Link, 1995, *supra*) animals transgenic for the *unc-54*/β(1-42) minigene construct contained muscle-specific deposits of anti-β peptide immunoreactivity. Non anti-β-immunoreactivity was observed in control animals (*i.e.*, non-transgenic, wild-type *C. elegans* tested concurrently). Although *C. elegans* is reported to contain a homolog of the β-amyloid precursor protein gene (*See*, Daigle and Li, Proc. Natl. Acad. Sci., 90:2045-2049 [1993]), this sequence does not contain an apparent β-peptide domain. Thus, it would not be expected, nor did it show, cross-reactivity with the anti-β antibody used in these experiments. For dually transgenic animals, immunoreactive deposits accumulated in the body wall of the animals.

Next, to determine whether the immunoreactive deposits observed in the *unc-54*/β-(1-42) strains displayed the tinctural properties of classic insoluble β-amyloid, transgenic

strains were fixed and stained with thioflavin S, a fluorescent amyloid-specific dye, as known in the art, and described by Guntern and Bouras (R. Guntern and C. Bouras, Experientia 48:-10 [1992]). Thioflavin S-reactive deposits were found in all strains containing the *unc-54*/β-(1-42) minigene constructs, but not in control wild-type animals.

Whole mounts of fixed dual transgenic animals stained with coumarin-phallacidin (a muscle-specific probe) and anti-β peptide antibody. Muscle-specific β peptide deposits were observed, and showed a qualitative correlation with the level of GFP expression in these muscle cells (*i.e.*, cells that contained more β-peptide deposits were more green than cells with less β-peptide deposits).

In addition, Congo Red and Chrysamine G, two dyes known to interact with β-amyloid and have been reported to interfere with its aggregation are tested on the dually transgenic animals. Anti-oxidants (*e.g.*, vitamin E and ascorbate) are also tested. In these experiments, interference with aggregation of β-peptide, or interaction with β-amyloid are observed.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

CLAIMS

What is claimed is:

1. A transparent animal comprising:

(a) a toxic transgene;

(b) an inducible promotor operably linked to a reporter gene, wherein the toxic transgene induces the promotor and wherein the expression of the reporter gene is detectable *in vivo*.

2. The transparent animal of claim 1, wherein the inducible promoter operably linked to the reporter gene is stably integrated into the genome of the animal.

3. The transparent animal of claim 2, wherein the inducible promotor is induced by disruption of cellular function.

4. The transparent animal of claim 2, wherein the inducible promoter is a heat shock promoter.

5. The transparent animal of claim 2, wherein the toxic transgene is β-peptide.

6. The transparent animal of claim 2, wherein said transparent animal is selected from the class Nematoda.

7. The transparent animal of Claim 6, wherein said transparent animal is *Caenorhabditis elegans*.

8. The transparent animal of Claim 2, wherein said genome comprises SEQ ID NO:5 and SEQ ID NO:8.

9. The transparent animal of Claim 2, wherein said reporter gene is green fluorescence protein (GFP).

10. The transparent animal of claim 1, further comprising a heterologous gene marker.

11. A method for producing a dually transgenic non-human animal comprising:

a) providing:

i) a first and second non-human animal; and

ii) a first toxic transgene; and

iii) a second transgene, comprising a reporter;

b) introducing said first toxic transgene into the genome of said first non-human animal to produce a first transgenic animal, and introducing said second transgene into the genome of said second non-human animal to produce a second transgenic animal; and

c) mating said first transgenic animal with said second transgenic animal to produce a dually transgenic animal, wherein said toxic transgene and said reporter are expressed.

12. A dually transgenic non-human animal produced according to the method of claim 11, wherein the toxic transgene is β-peptide.

13. A dually transgenic non-human animal produced according to the method of claim 11, wherein said non-human animal is transparent.

14. The dually transgenic non-human animal of claim 13, wherein said non-human animal is a nematode.

15 The dually transgenic non-human animal of claim 14, wherein said nematode is *Caenorhabditis elegans*

16. The method of claim 11, wherein said reporter is green fluorescent protein.

17. The dually transgenic non-human animal produced according to the method of claim 11, wherein said first transgene comprises pCL25.

18. The dually transgenic non-human animal produced according to the method of claim 11, wherein said second transgene comprises pCL12.

19. The dually transgenic non-human animal produced according to the method of claim 11, further comprising a heterologous marker gene.

20. A method for testing compounds for toxicity, comprising:

a) providing:

i) a dually transgenic non-human animal expressing a toxic transgene and reporter gene operably linked to a promoter inducible by the toxic transgene;

ii) a composition comprising a test compound in a form suitable for administration such that said compound is bioavailable in the cells of said non-human animal; and

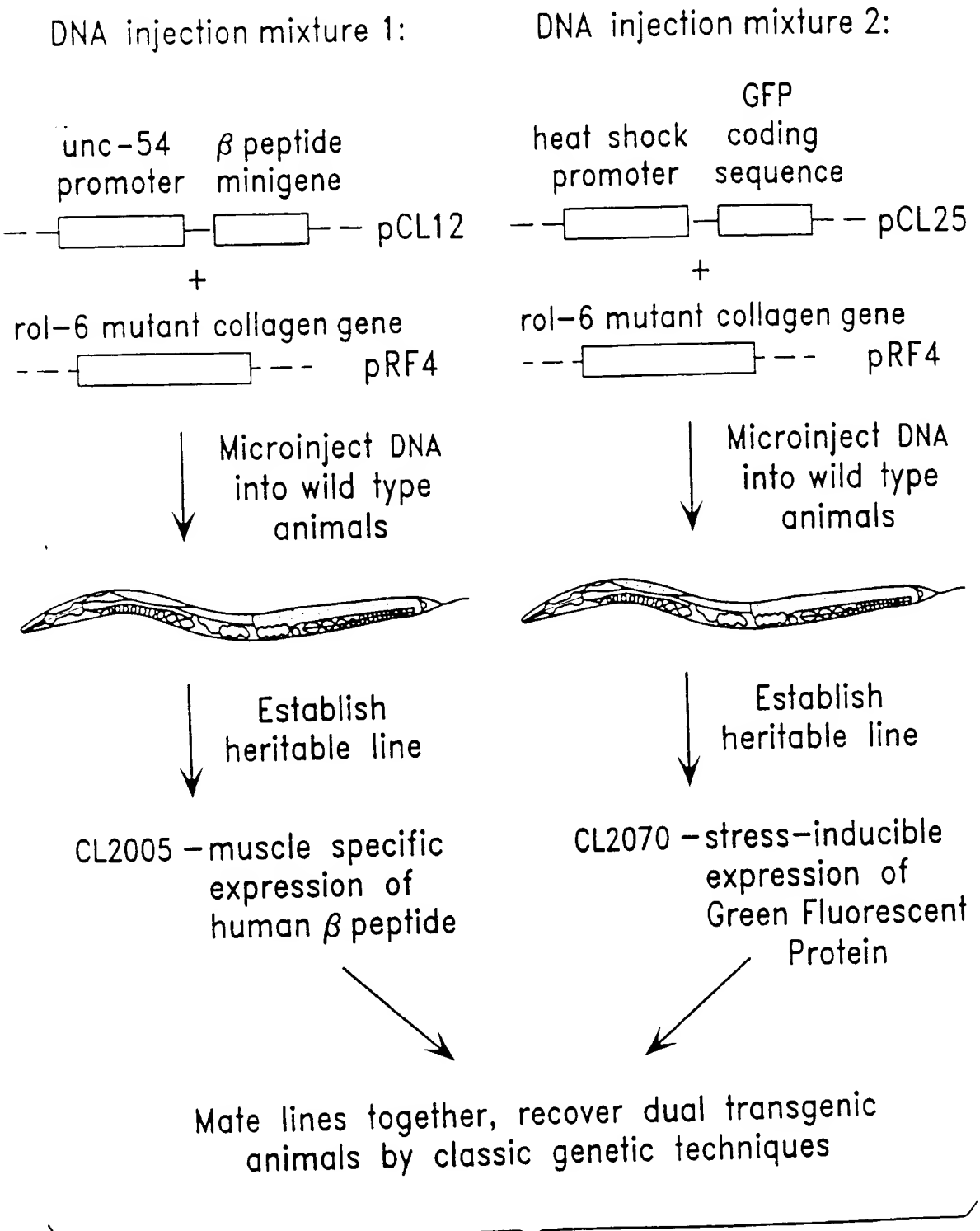
b) administering said test compound to said non-human animal.

21. The method according to claim 20, wherein the toxic transgene is β-peptide and the reporter gene is GFP.

22. The method of Claim 21, further comprising c) measuring a reduction in the fluorescence of said non-human animal and thereby identifying a compound as therapeutic.

23. The method of Claim 20, wherein said compounds inactivate said β-peptide expressed by said dually transgenic animal.

SUBSTITUTE SHEET (RULE 26)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+
rol-6 mutant collagen gene
pRF4
Microinject DNA into wild type animals
Establish heritable line
CL2005 – muscle specific expression of human β peptide
DNA injection mixture 2:
heat shock promoter
GFP coding sequence
pCL25
+
rol-6 mutant collagen gene
pRF4
Microinject DNA into wild type animals
Establish heritable line
CL2070 – stress-inducible expression of Green Fluorescent Protein
Mate lines together, recover dual transgenic animals by classic genetic techniques

Fig. 1

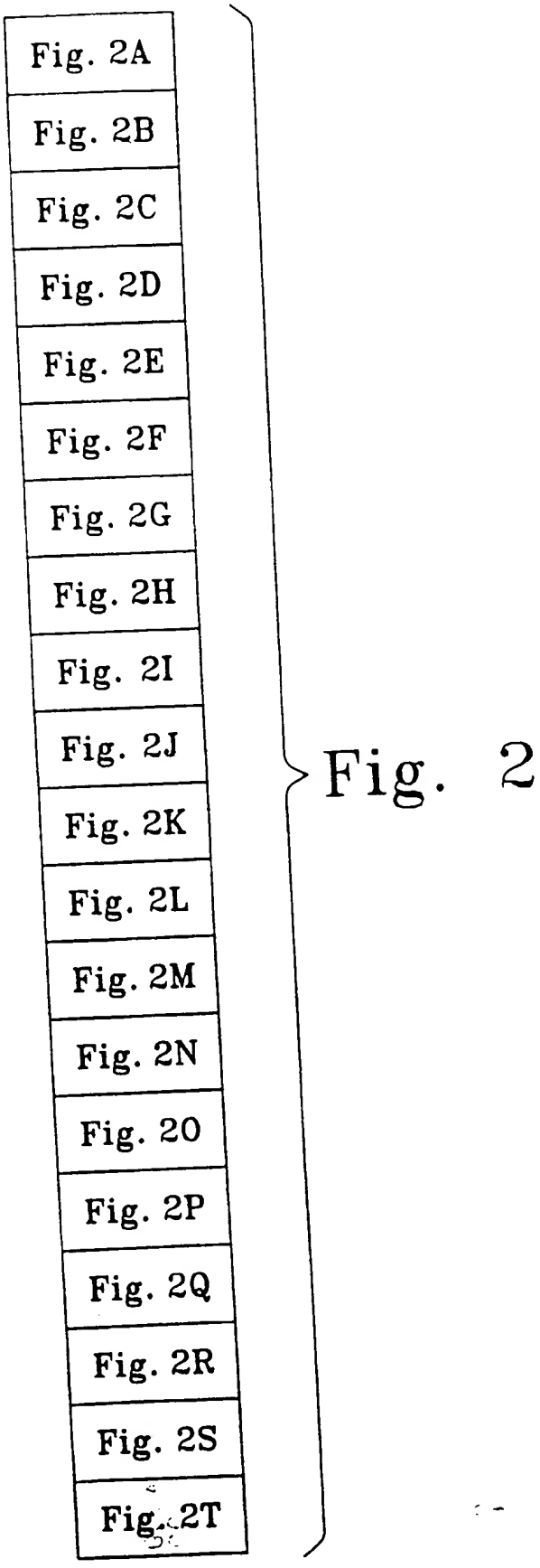
Fig. 2A
Fig. 2B
Fig. 2C
Fig. 2D
Fig. 2E
Fig. 2F
Fig. 2G
Fig. 2H
Fig. 2I
Fig. 2J
Fig. 2K
Fig. 2L
Fig. 2M
Fig. 2N
Fig. 2O
Fig. 2P
Fig. 2Q
Fig. 2R
Fig. 2S
Fig. 2T

Fig. 2

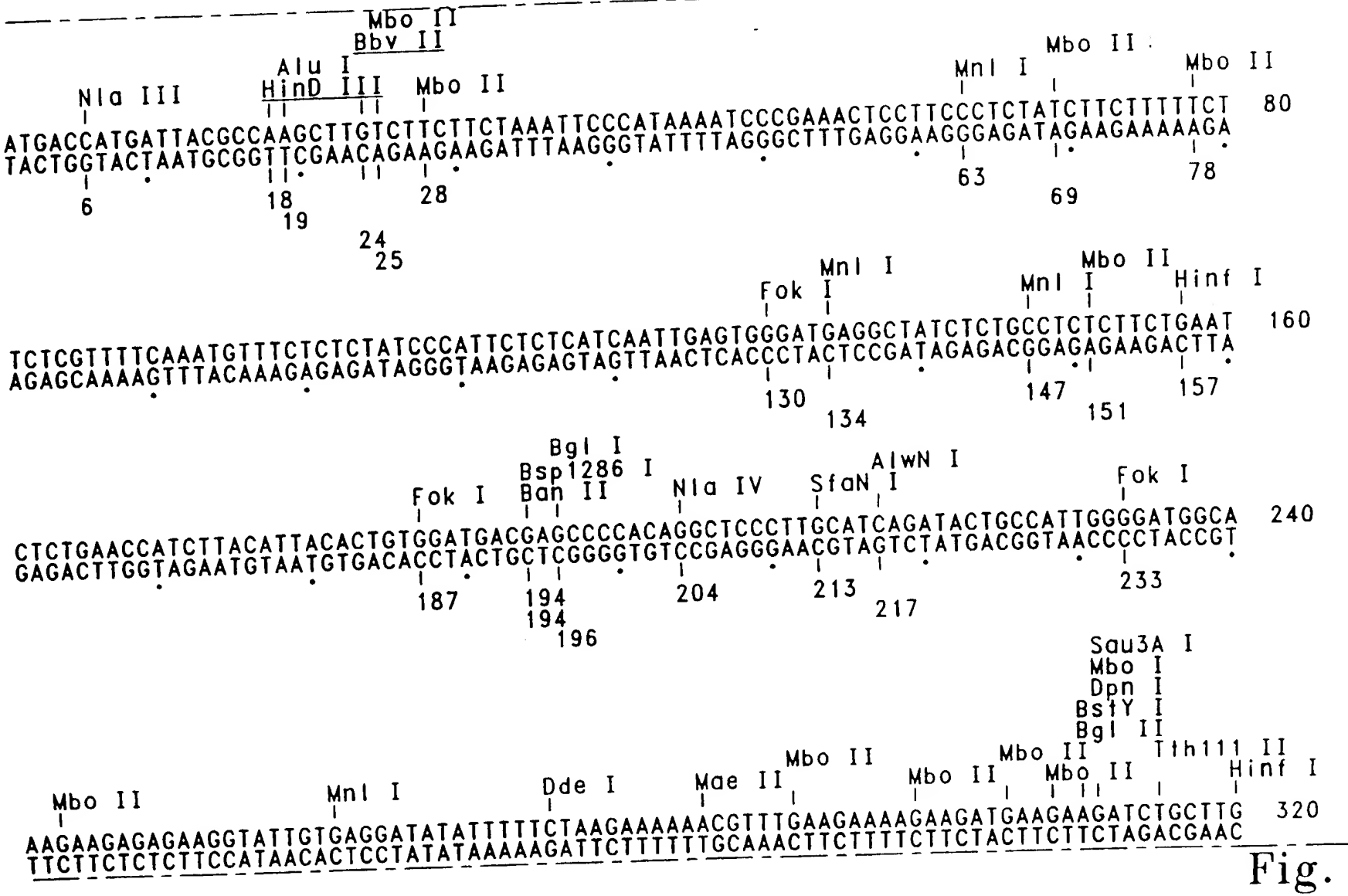
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Fig. 2A

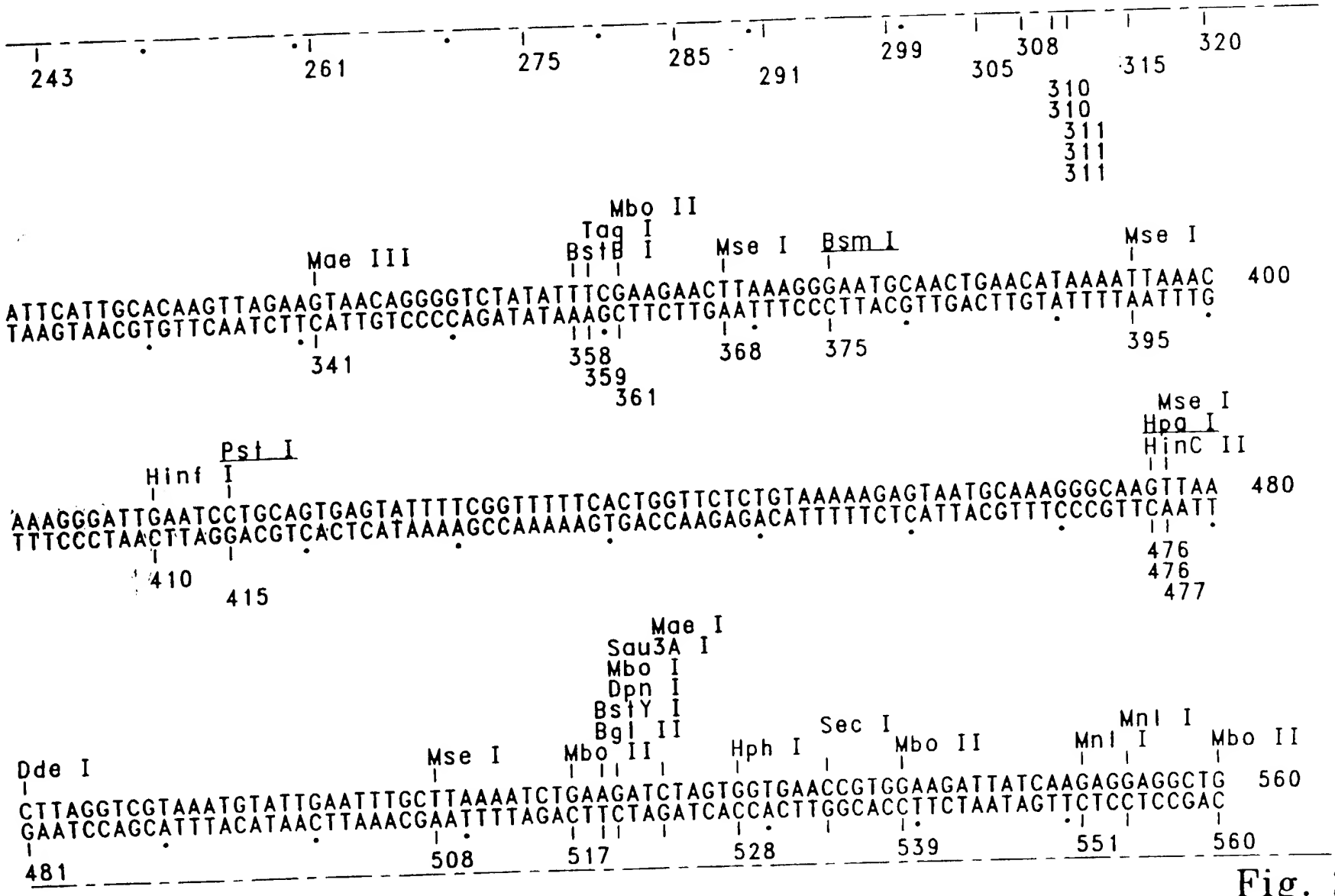
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Fig. 2B

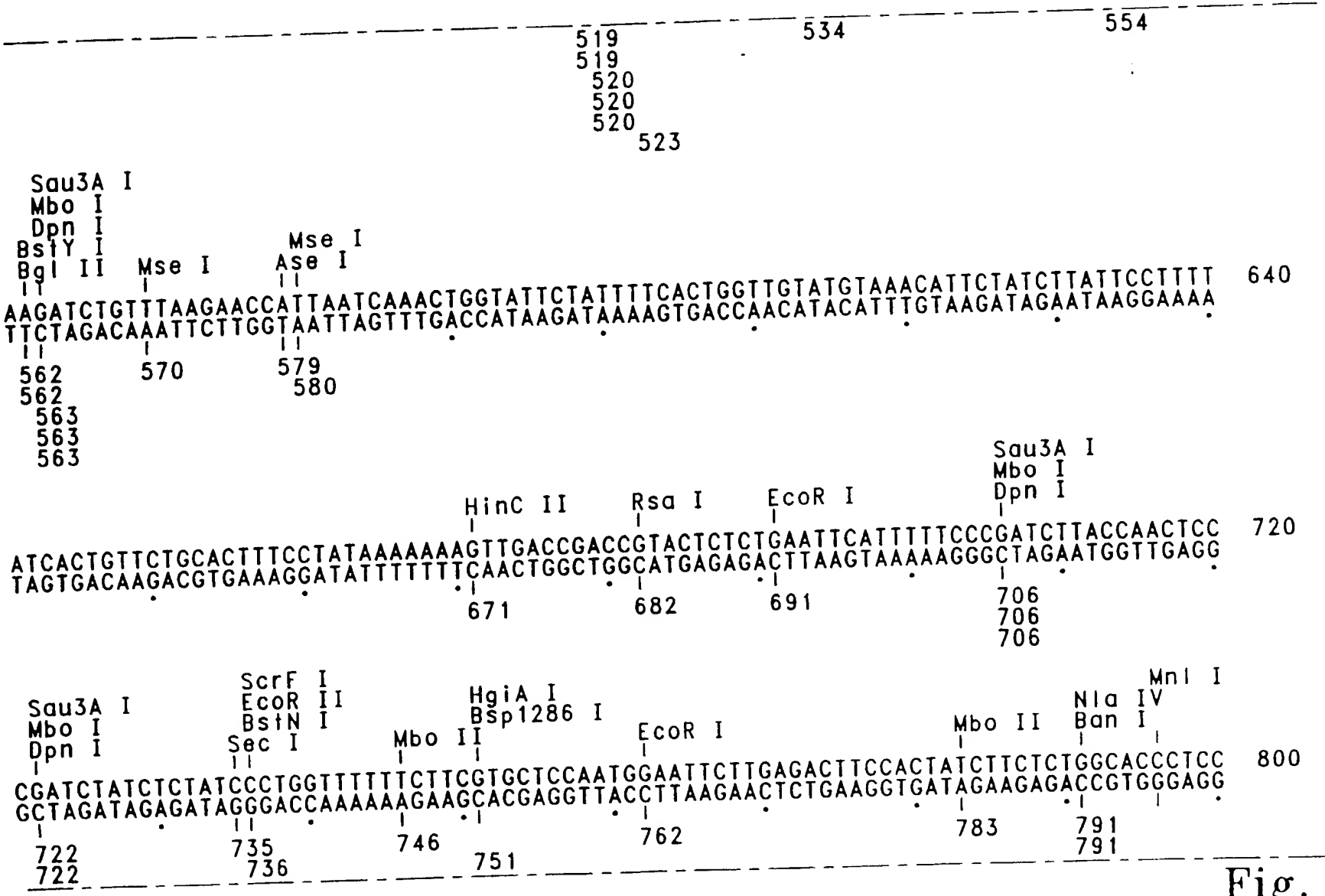
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Fig. 2C

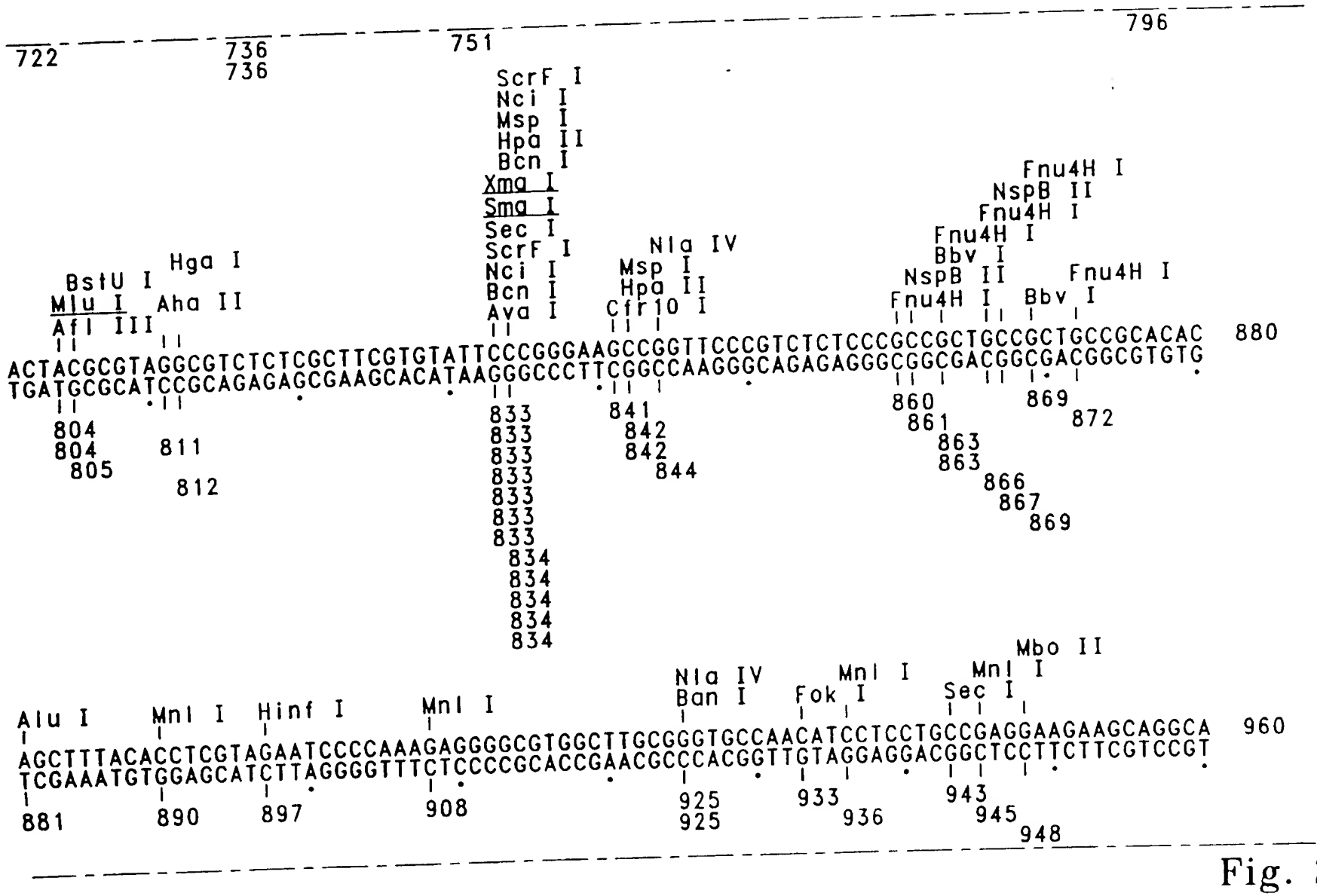
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Fig. 2D

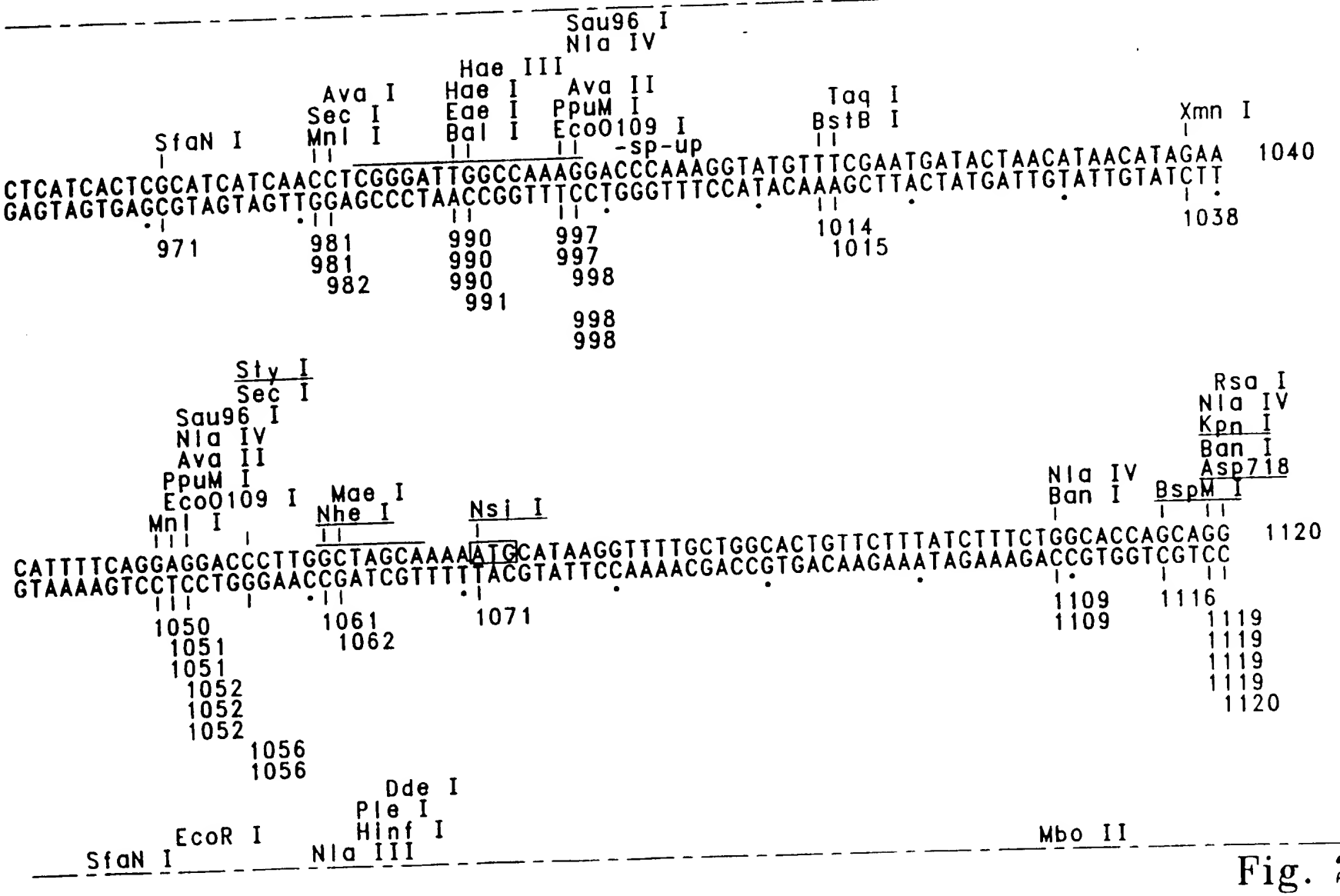
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Fig. 2E

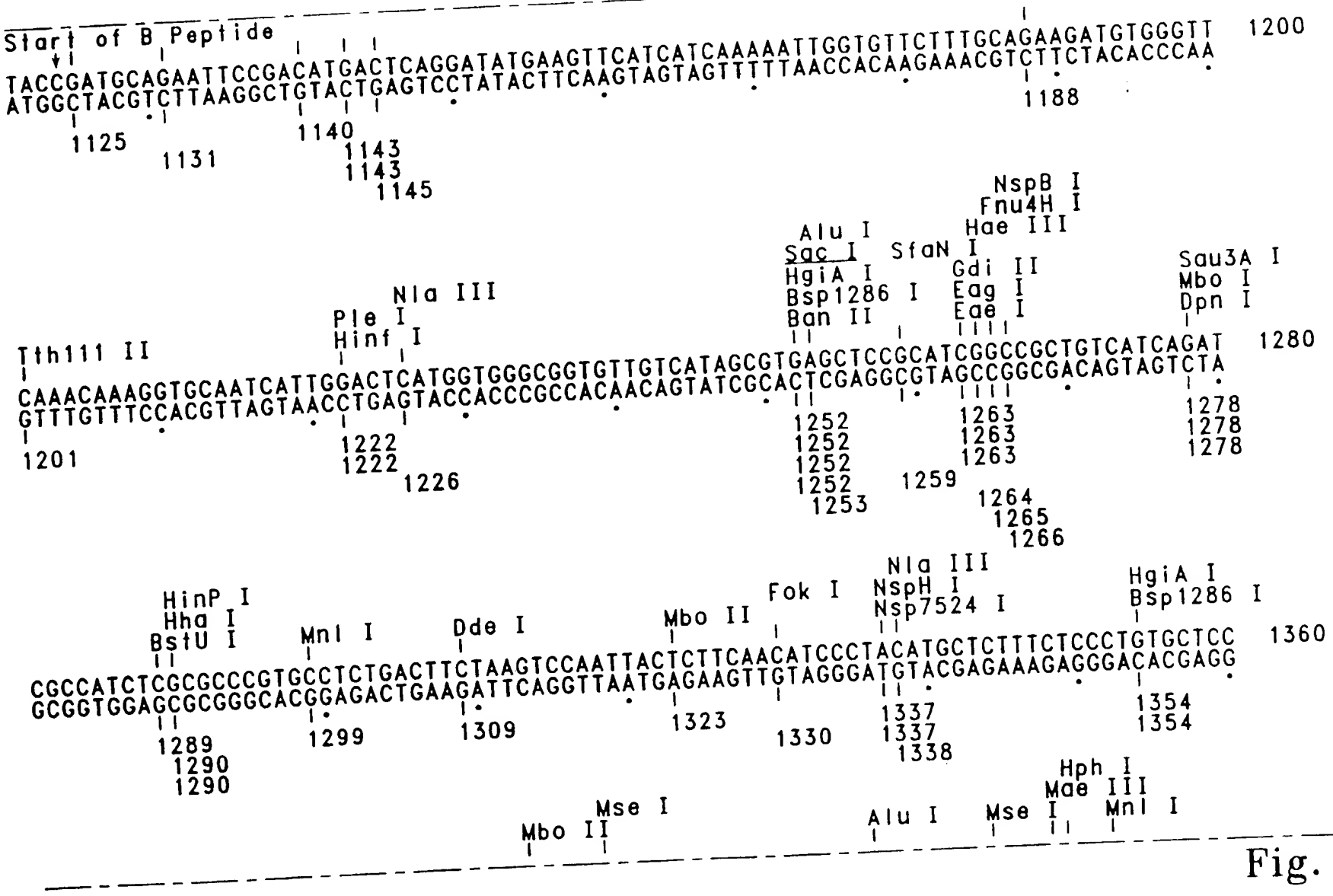
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Fig. 2F

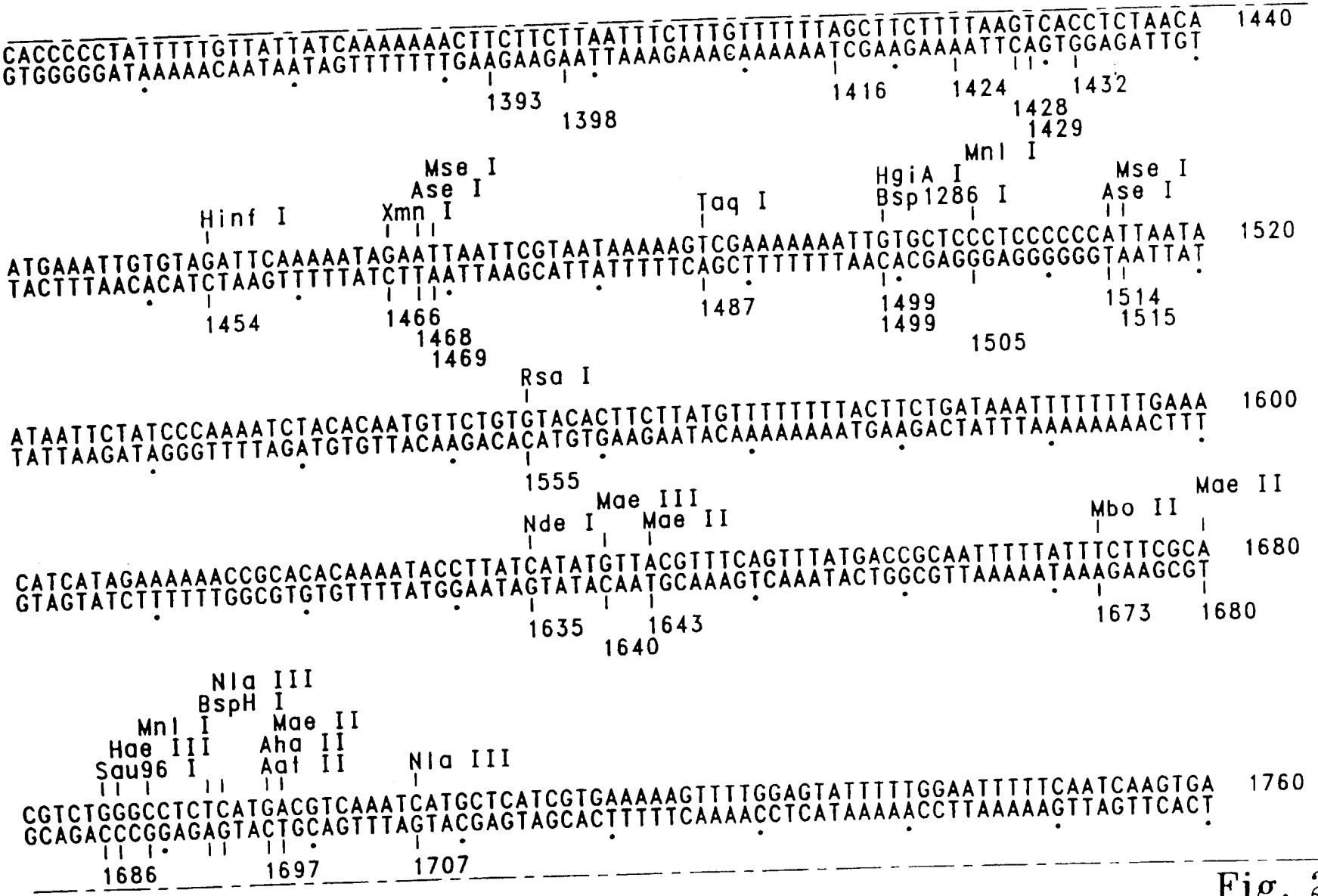
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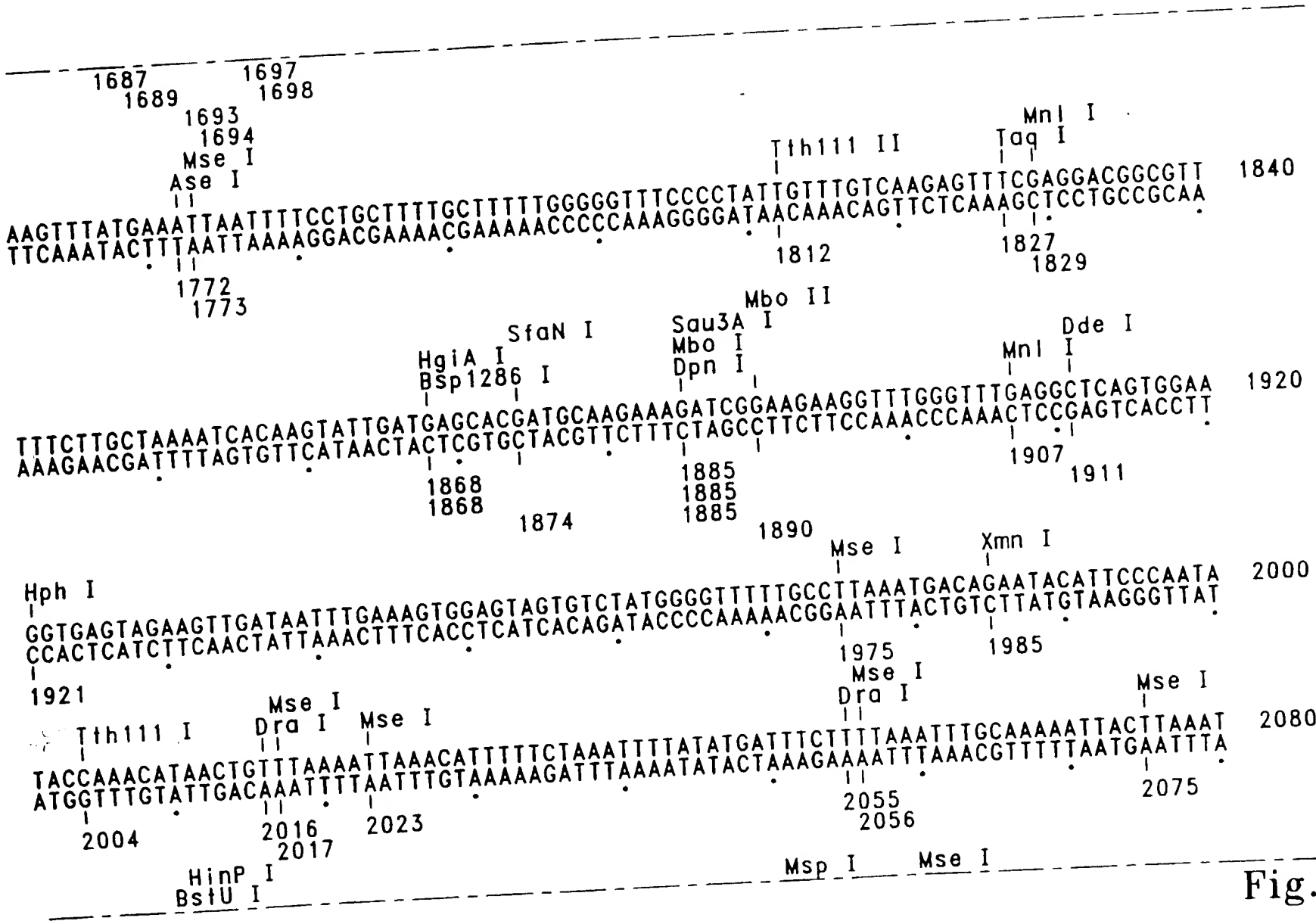
Fig. 2G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CCACTCATCTTCAACTATTAAACTTTCACCTCATCACAGATACCCCAAAAACGGAATTTACTGTCTTATGTAAGGGTTAT
1921
1975
1985
Mse I
Dra I
Mse I
Tth111 I
Mse I
Dra I
Mse I
2080
TACCAAACATAACTGTTTAAAATTAAACATTTTTCTAAATTTTATATGATTTCTTTTAAATTTGCAAAAATTACTTAAAT
ATGGTTTGTATTGACAAATTTTAATTTGTAAAAAGATTTAAAATATACTAAAGAAAATTTAAACGTTTTTAATGAATTTA
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2016
2017
2023
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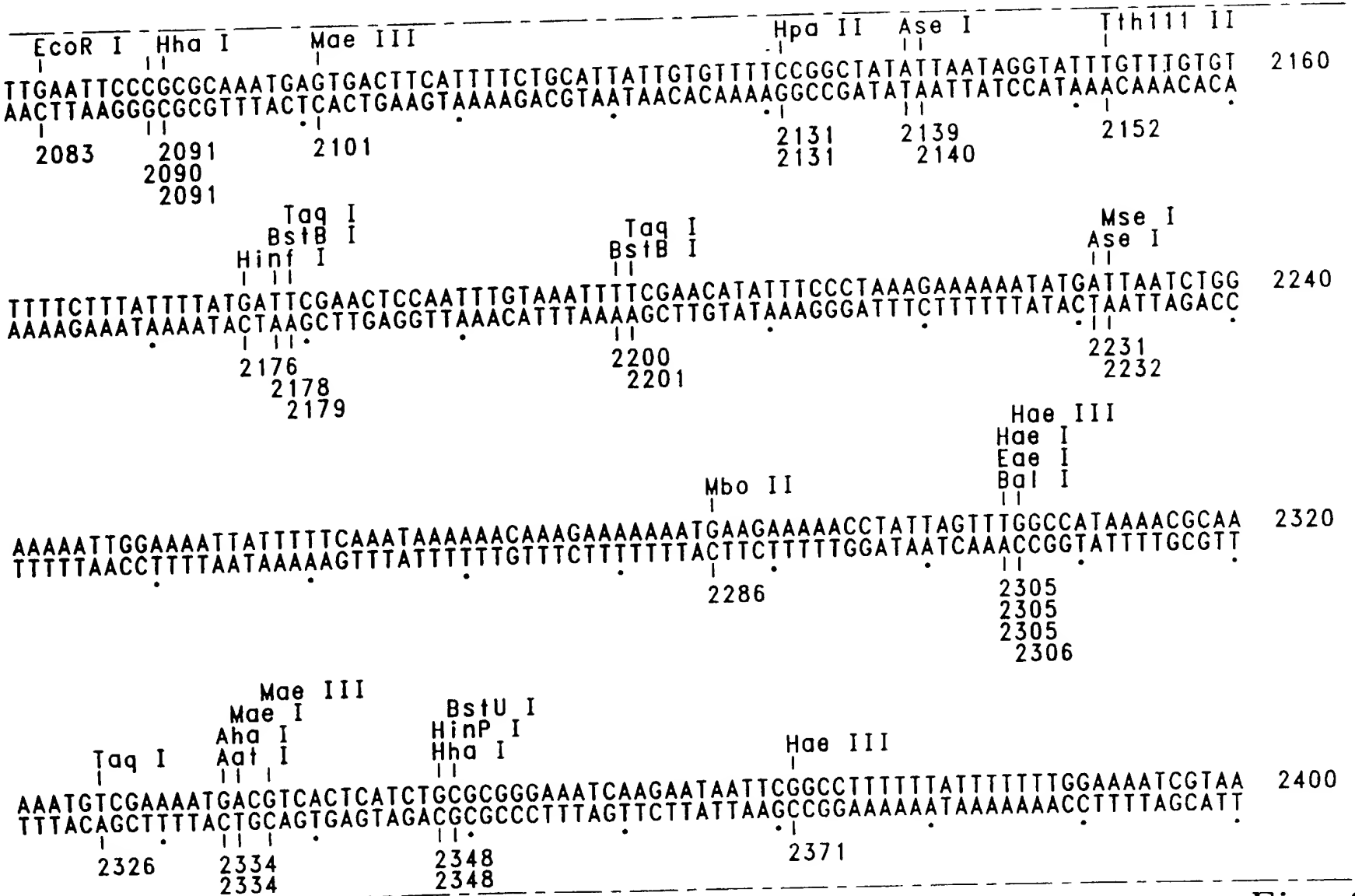
2075
HinP I
BstU I
Msp I
Mse I

Fig. 2H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Hha I
Mae III
Hpa II
Ase I
Tth111 II
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2083
2091
2090
2091
2101
2131
2131
2139
2140
2152
Taq I
BstB I
Hinf I
Taq I
BstB I
Mse I
Ase I
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2176
2178
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2200
2201
2231
2232
Hae III
Hae I
Eae I
Bal I
Mbo II
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2286
2305
2305
2305
2306
Mae III
Mae I
Aha I
Aat I
Taq I
BstU I
HinP I
Hha I
Hae III
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TTTACAGCTTTTACTGCAGTGAGTAGACGCGCCCTTTAGTTCTTATTAAGCCGGAAAAAATAAAAAAACCTTTTAGCATT
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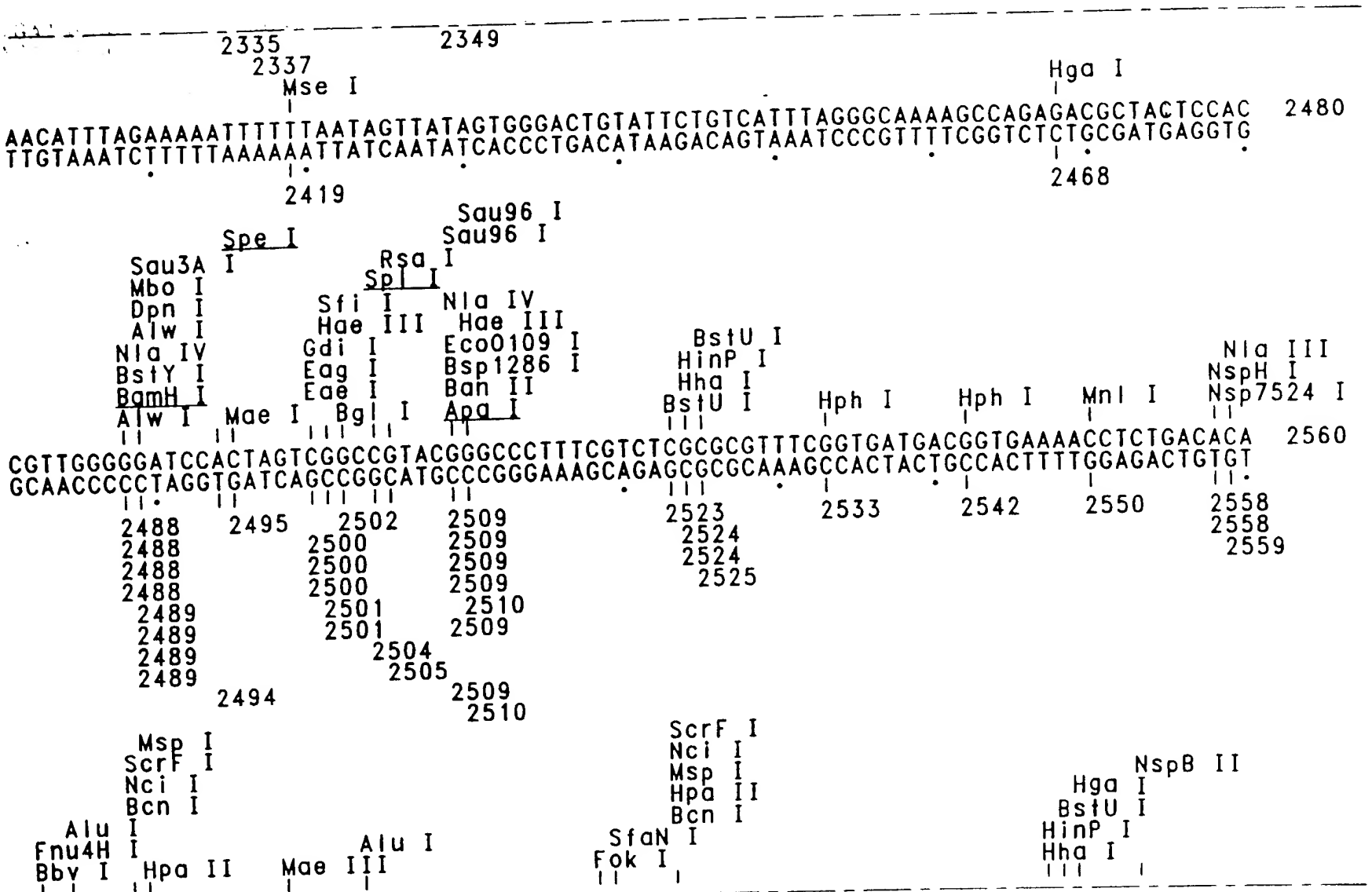
2371

Fig. 2I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Hga I
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TTGTAAATCTTTTTAAAAAATTATCAATATCACCCTGACATAAGACAGTAAATCCCGTTTTCGGTCTCTGCGATGAGGTG
2419
2468
Sau96 I
Spe I
Sau96 I
Rsa I
Sau3A I
Spl I
Mbo I
Dpn I
Sfi I
Nla IV
Alw I
Hae III
Hae III
Gdi I
Eco0109 I
BstU I
Nla IV
Eag I
Bsp1286 I
HinP I
Nla III
BstY I
Eae I
Ban II
Hha I
NspH I
BamH I
Alw I
Mae I
Bgl I
Apa I
BstU I
Hph I
Hph I
Mnl I
Nsp7524 I
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2500
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2500
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2489
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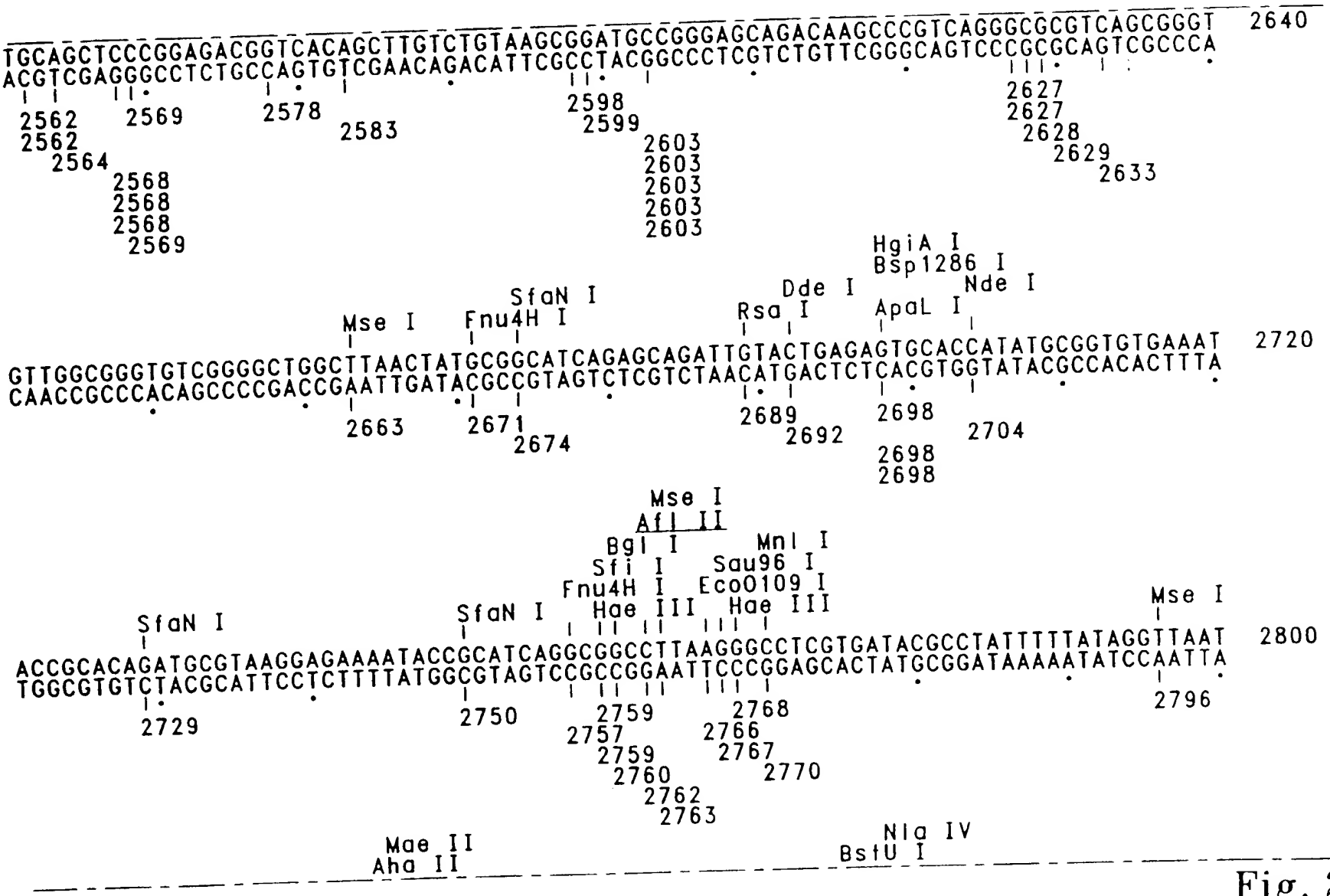
2509
2510
Msp I
ScrF I
ScrF I
Nci I
Nci I
Msp I
Hpa II
Bcn I
Bcn I
NspB II
Hga I
Alu I
BstU I
Alu I
HinP I
Fnu4H I
SfaN I
Hha I
Bby I
Hpa II
Mae III
Fok I

Fig. 2J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2562 2564 2568 2568 2568 2569
HgiA I
Bsp1286 I
SfaN I
Dde I
Nde I
Mse I
Fnu4H I
Rsa I
ApaL I
GTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAAT 2720
CAACCGCCCACAGCCCCGACCGAATTGATACGCCGTAGTCTCGTCTAACATGACTCTCACGTGGTATACGCCACACTTTA
2663 2671 2674 2689 2692 2698 2698 2698 2704
Mse I
Afl II
Bgl I
Mnl I
Sfi I
Sau96 I
Fnu4H I
Eco0109 I
SfaN I
SfaN I
Hae III
Hae III
Mse I
ACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGGCCTTAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAAT 2800
TGGCGTGTCTACGCATTCCTCTTTTATGGCGTAGTCCGCCGGAATTCCCGGAGCACTATGCGGATAAAAATATCCAATTA
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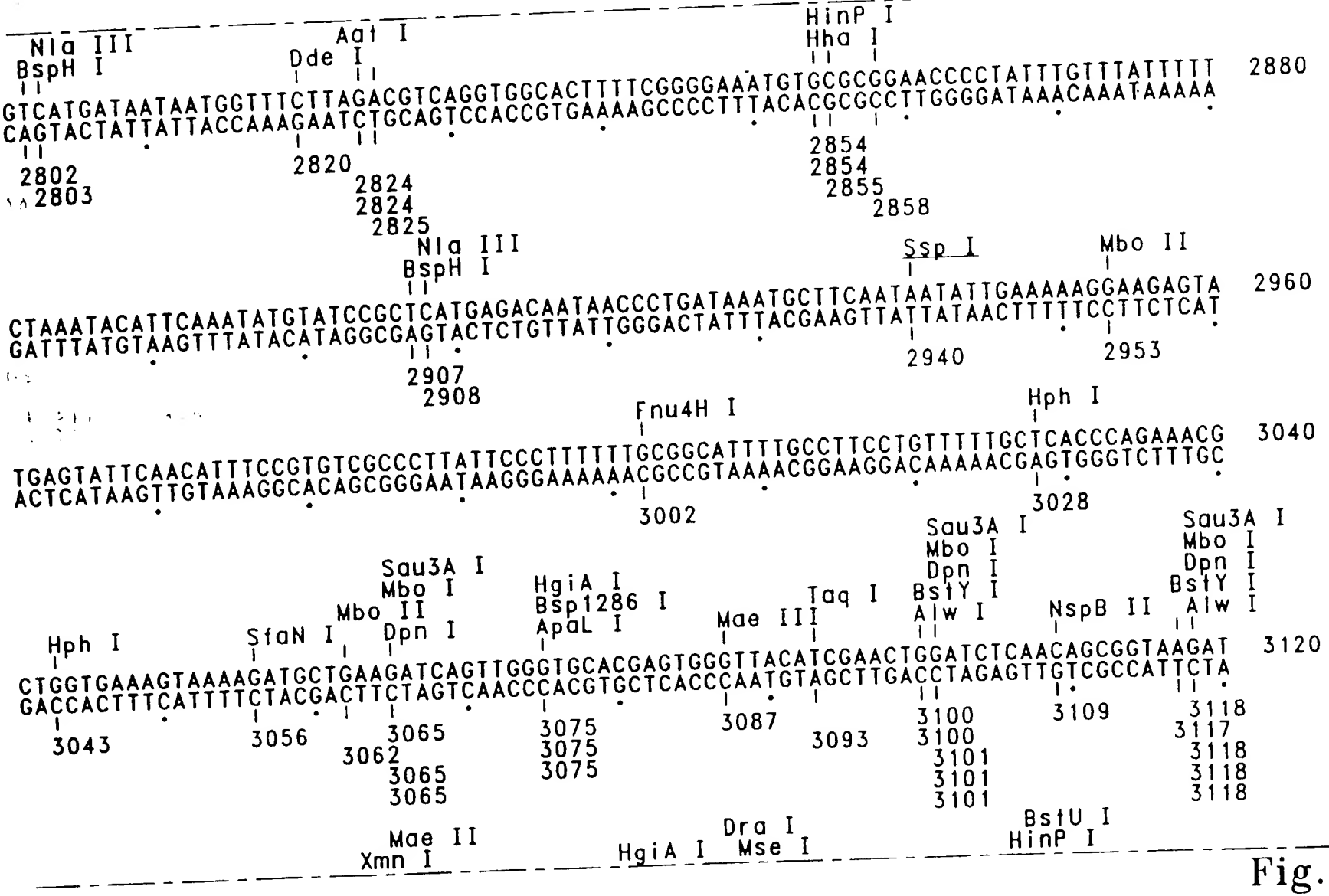
Nla IV
Mae II
Aha II
BstU I

Fig. 2K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BspH I
Aat I
Dde I
HinP I
Hha I
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2802
2803
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2824
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2854
2854
2855
2858
Nla III
BspH I
Ssp I
Mbo II
2960
2907
2908
2940
2953
Fnu4H I
Hph I
3040
3002
3028
Sau3A I
Mbo I
Dpn I
Sau3A I
Mbo I
Dpn I
BstY I
Alw I
Sau3A I
Mbo I
Dpn I
BstY I
Alw I
Mbo II
HgiA I
Bsp1286 I
ApaL I
Taq I
Mae III
NspB II
Hph I
SfaN I
Dpn I
3120
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3101
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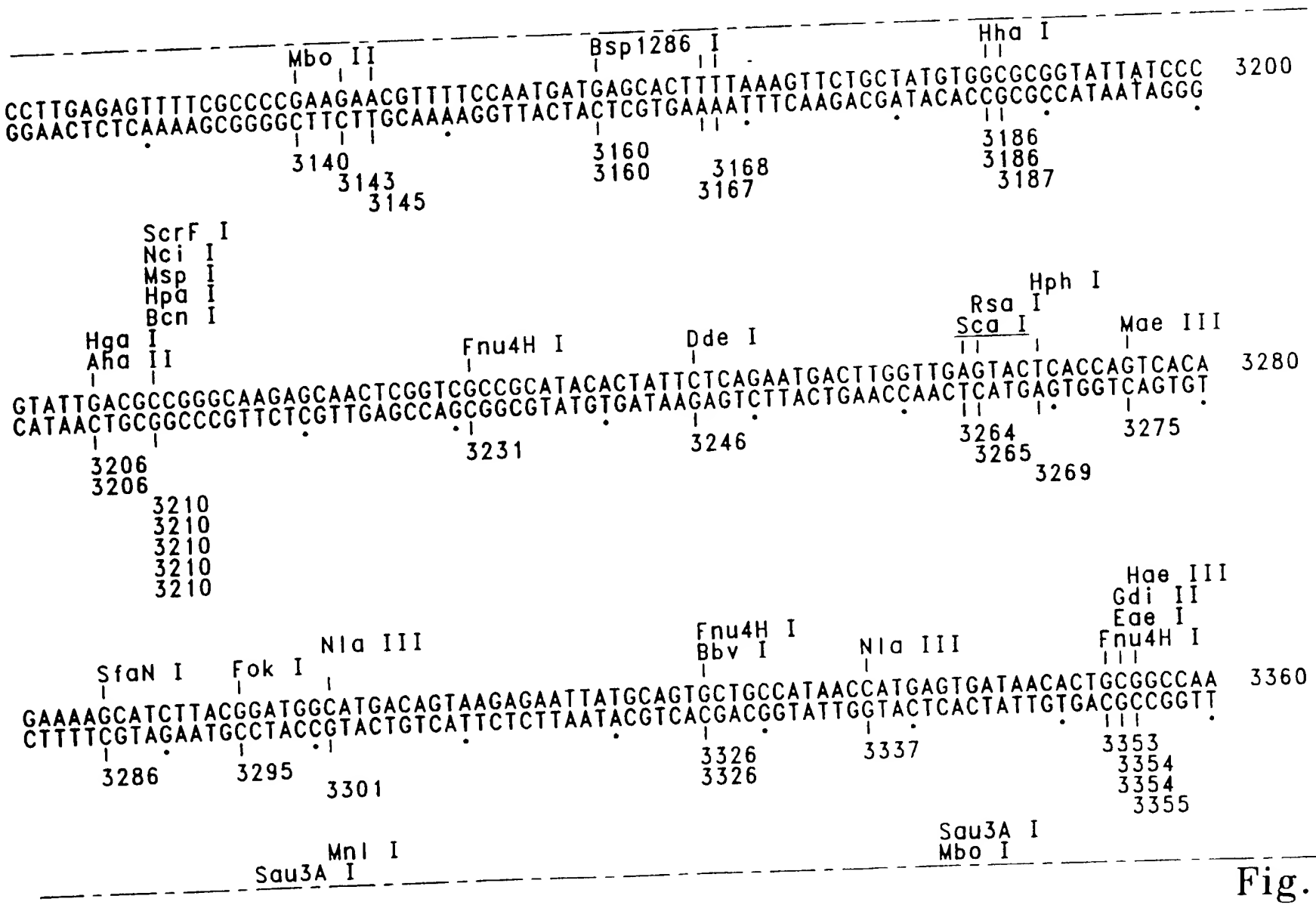
Xmn I
HgiA I
Dra I
Mse I
BstU I
HinP I

Fig. 2L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3160
3168
3167
3186
3186
3187
ScrF I
Nci I
Msp I
Hpa I
Bcn I
Hga I
Aha II
Fnu4H I
Dde I
Hph I
Rsa I
Sca I
Mae III
3280
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3206
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3231
3246
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3265
3269
3275
Hae III
Gdi II
Eae I
Fnu4H I
Nla III
Fnu4H I
Bbv I
Nla III
SfaN I
Fok I
3360
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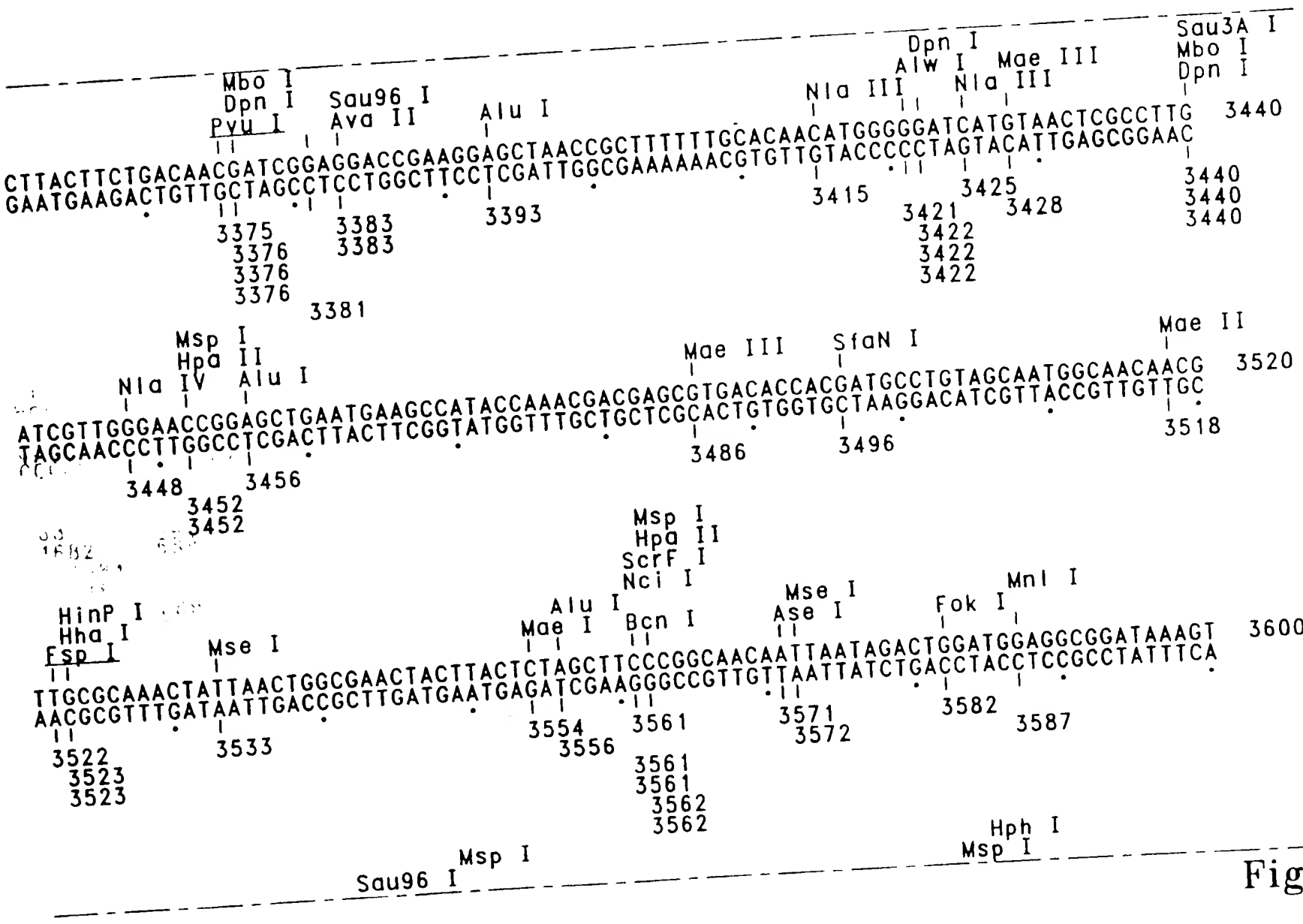
Sau3A I
Mbo I
Mnl I
Sau3A I

Fig. 2M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Dpn I
Pvu I
Sau96 I
Ava II
Alu I
Nla III
Dpn I
Alw I
Mae III
Nla III
Sau3A I
Mbo I
Dpn I
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GAATGAAGACTGTTGCTAGCCTCCTGGCTTCCTCGATTGGCGAAAAAACGTGTTGTACCCCCTAGTACATTGAGCGGAAC
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Msp I
Hpa II
Nla IV
Alu I
Mae III
SfaN I
Mae II
ATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACG 3520
TAGCAACCCTTGGCCTCGACTTACTTCGGTATGGTTTGCTGCTCGCACTGTGGTGCTAAGGACATCGTTACCGTTGTTGC
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Msp I
Hpa II
ScrF I
Nci I
Alu I
Mae I
Bcn I
Mse I
Ase I
Fok I
Mnl I
HinP I
Hha I
Fsp I
Mse I
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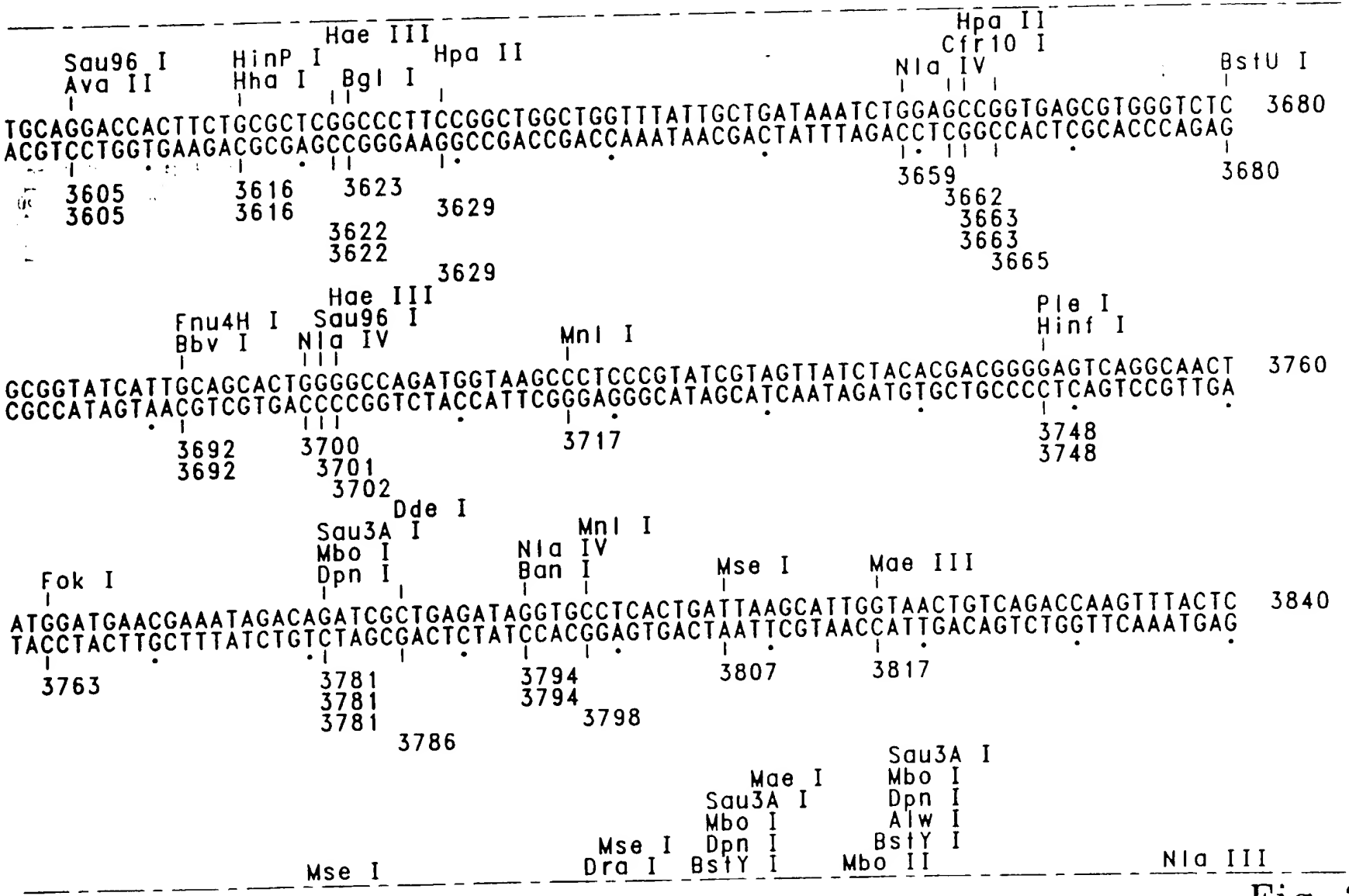
Hph I
Msp I
Msp I
Sau96 I

Fig. 2N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Hpa II
Cfr10 I
Sau96 I
Ava II
HinP I
Hha I
Bgl I
Nla IV
BstU I
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3662
3663
3663
3665
3680
Hae III
Fnu4H I
Bbv I
Sau96 I
Nla IV
Mnl I
Ple I
Hinf I
GCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACT 3760
CGCCATAGTAACGTCGTGACCCCGGTCTACCATTCGGGAGGGCATAGCATCAATAGATGTGCTGCCCCTCAGTCCGTTGA
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Dde I
Sau3A I
Mbo I
Dpn I
Mnl I
Nla IV
Ban I
Fok I
Mse I
Mae III
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Sau3A I
Mbo I
Dpn I
Alw I
BstY I
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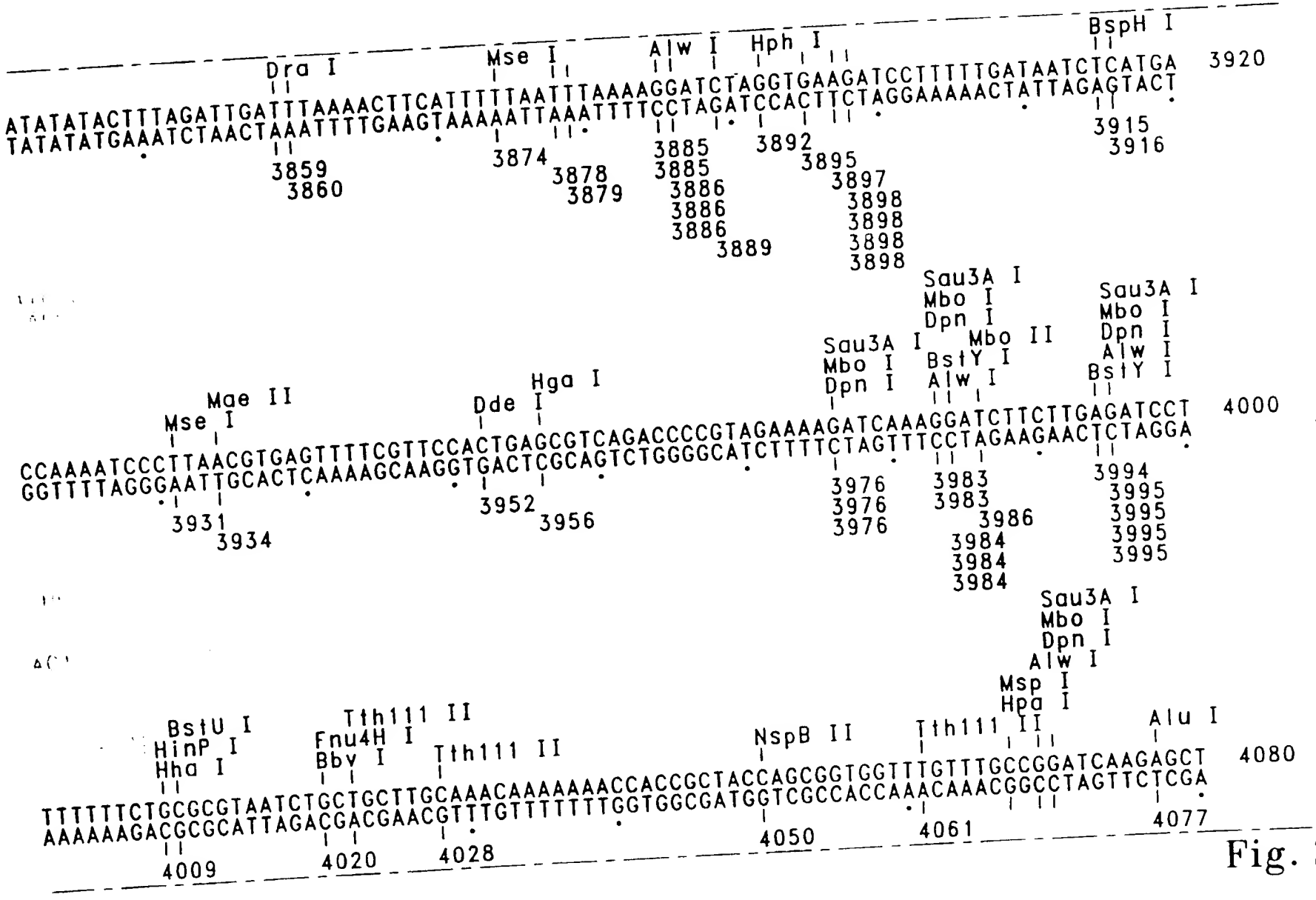
Dpn I
Mse I
Dra I
BstY I
Mbo II
Mse I
Nla III

Fig. 20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Mbo I
Dpn I
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Mbo I
Dpn I
Alw I
BstY I
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Mbo II
Sau3A I
Mbo I
Dpn I
BstY I
Alw I
Mae II
Mse I
Hga I
Dde I
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Sau3A I
Mbo I
Dpn I
Alw I
Msp I
Hpa I
BstU I
HinP I
Hha I
Tth111 II
Fnu4H I
Bbv I
Tth111 II
NspB II
Tth111 II
Alu I
4080
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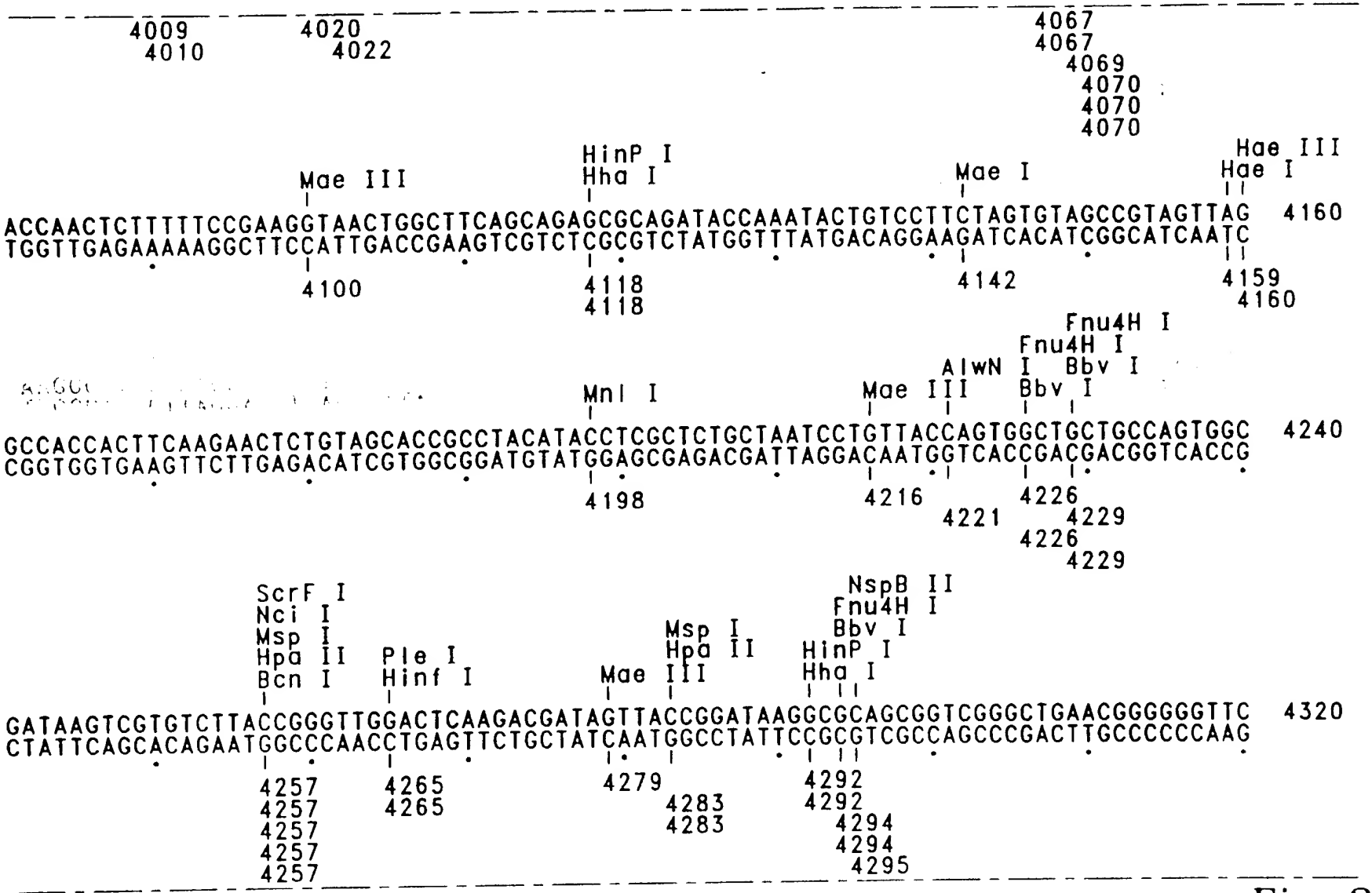
Fig. 2P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Mae III
Mae I
Hae III
Hae I
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4160
Fnu4H I
Fnu4H I
AlwN I
Bbv I
Mnl I
Mae III
Bbv I
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ScrF I
Nci I
Msp I
Hpa II
Bcn I
Ple I
Hinf I
Msp I
Hpa II
Mae III
NspB II
Fnu4H I
Bbv I
HinP I
Hha I
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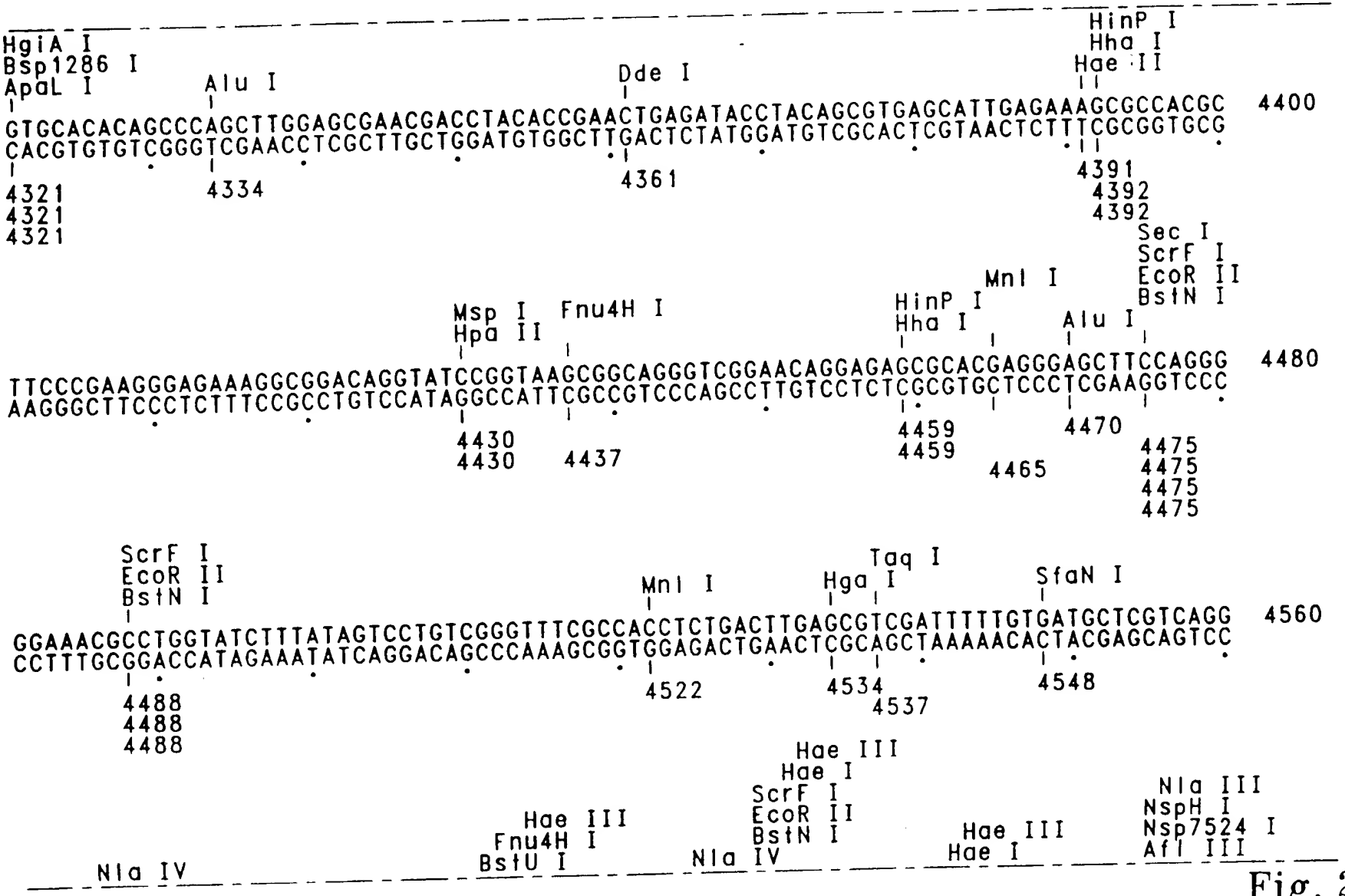
Fig. 2Q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Bsp1286 I
ApaL I
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Dde I
HinP I
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ScrF I
EcoR II
BstN I
Mnl I
Msp I
Hpa II
Fnu4H I
HinP I
Hha I
Alu I
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ScrF I
EcoR II
BstN I
Mnl I
Taq I
Hga I
SfaN I
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4537
4548
Hae III
Hae I
ScrF I
EcoR II
BstN I
Hae III
Fnu4H I
BstU I
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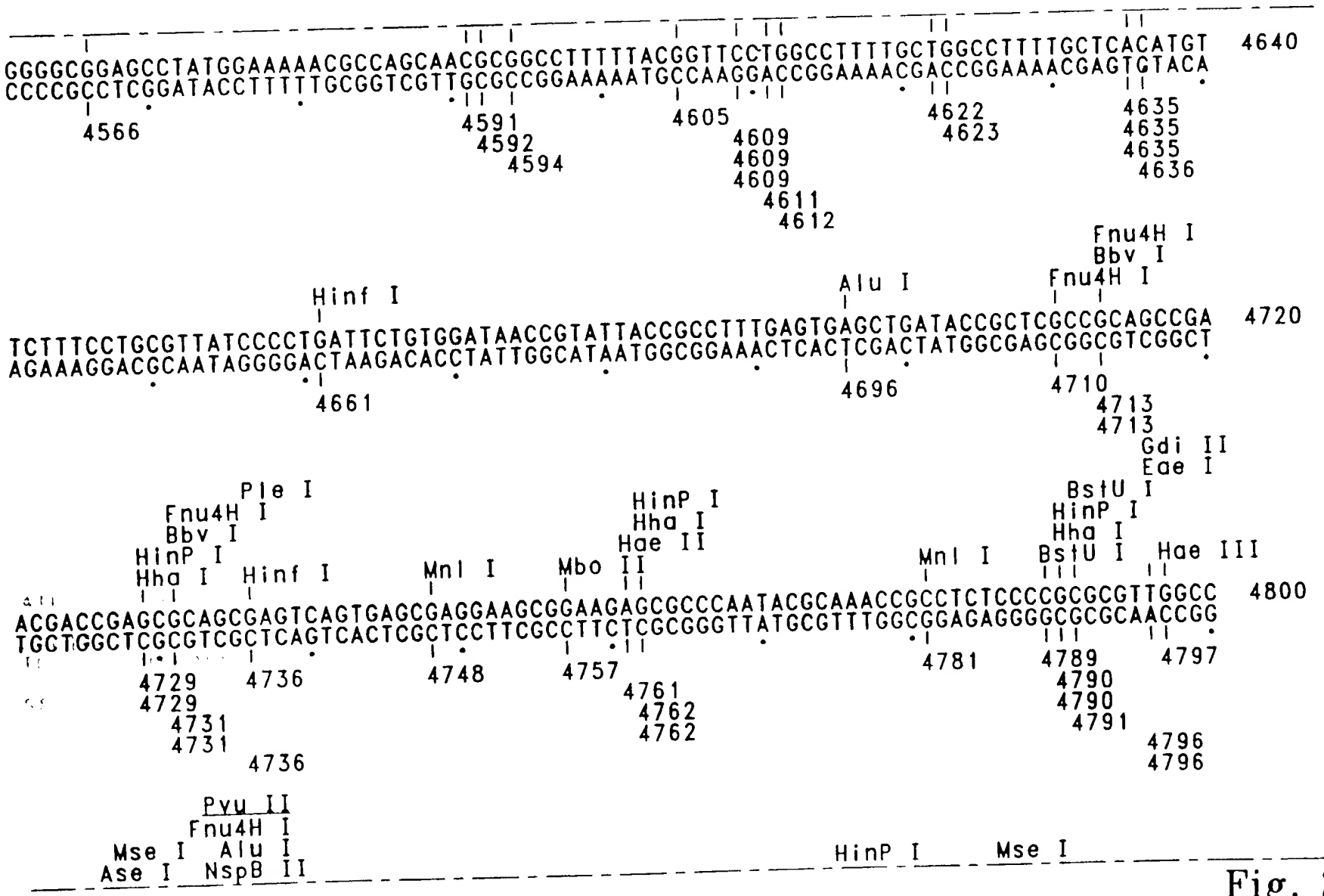
Nla IV
Hae III
Hae I
Nla III
NspH I
Nsp7524 I
Afl III

Fig. 2R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4636
Fnu4H I
Bbv I
Hinf I
Alu I
Fnu4H I
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4661
4696
4710
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4713
Gdi II
Eae I
Ple I
Fnu4H I
Bbv I
HinP I
Hha I
Hinf I
Mnl I
Mbo II
HinP I
Hha I
Hae II
Mnl I
BstU I
HinP I
Hha I
BstU I
Hae III
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4748
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4781
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4790
4790
4791
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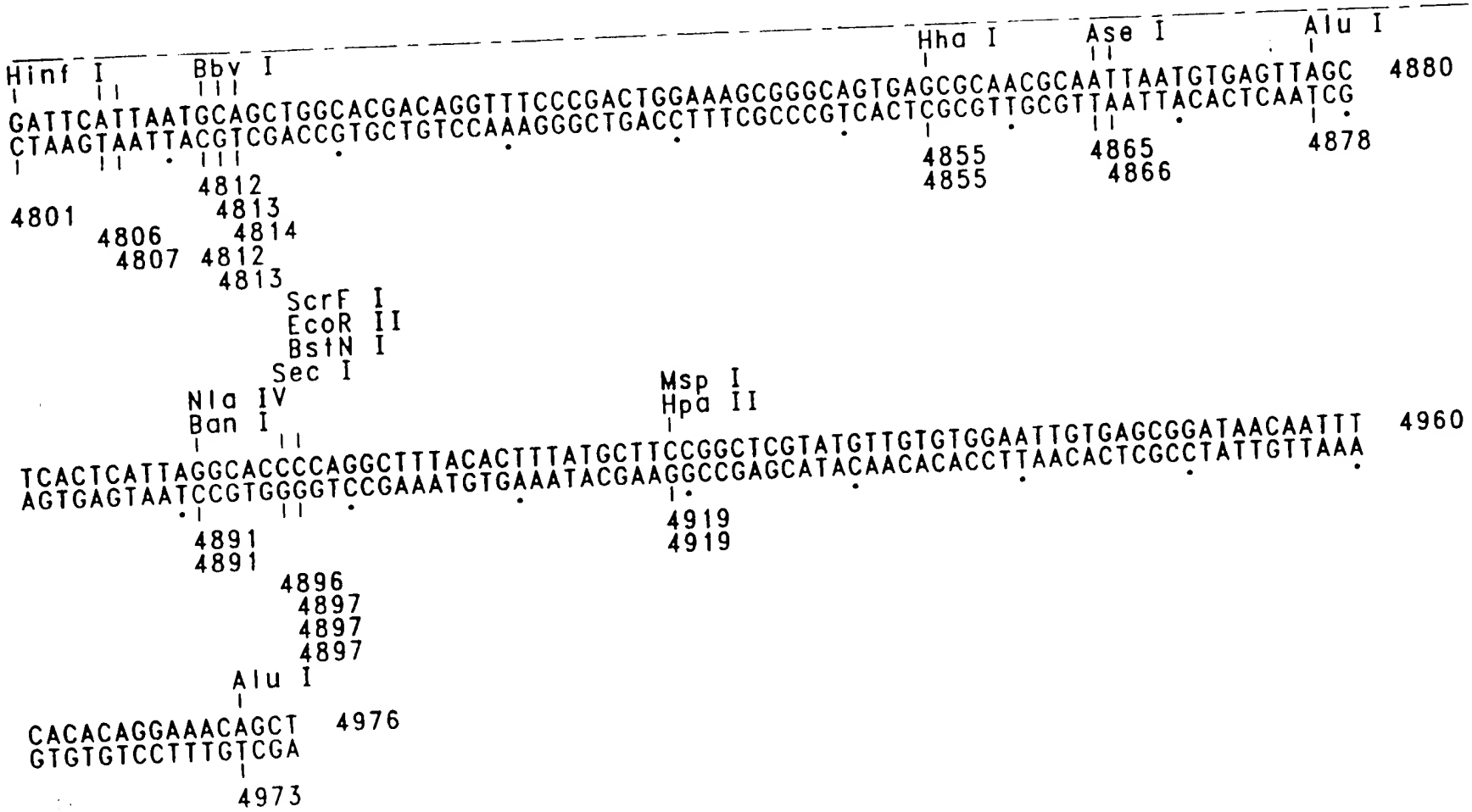
Pvu II
Fnu4H I
Mse I
Alu I
Ase I
NspB II
HinP I
Mse I

Fig. 2S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Ase I
Alu I
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4807
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ScrF I
EcoR II
BstN I
Sec I
Msp I
Hpa II
Nla IV
Ban I
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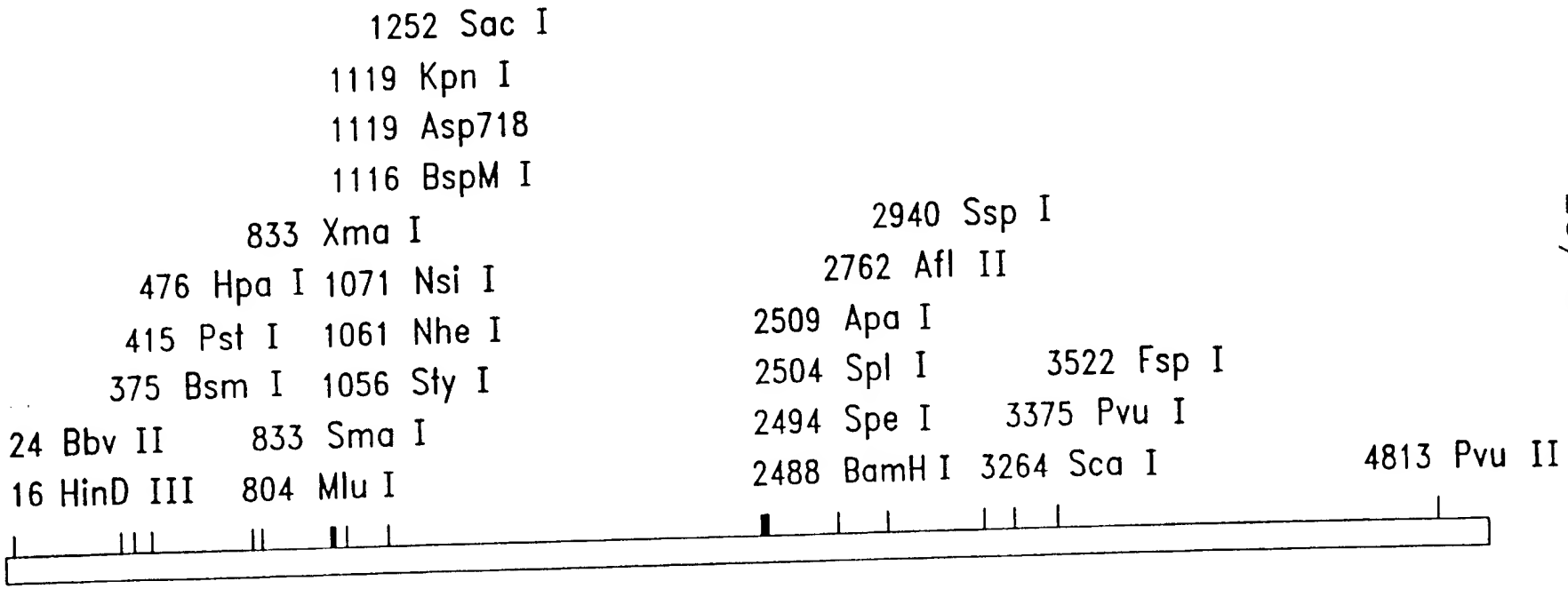
4897
Alu I
4976
4973

Fig. 2T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1119 Asp718
1116 BspM I
833 Xma I
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415 Pst I
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375 Bsm I
1056 Sty I
24 Bbv II
833 Sma I
16 HinD III
804 Mlu I
2940 Ssp I
2762 Afl II
2509 Apa I
2504 Spl I
3522 Fsp I
2494 Spe I
3375 Pvu I
2488 BamH I
3264 Sca I
4813 Pvu II

Fig. 3

```
1071 /    1                                                1101 /   11
ATG CAT AAG GTT TTG CTG GCA CTG TTC TTT ATC TTT CTG GCA CCA GCA GGT ACC GAT GCA
met his lys val leu leu ala leu phe phe ile phe leu ala pro ala gly thr asp ala
1131 /   21                                               1161 /   31
GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT CAA AAA TTG GTG TTC TTT GCA GAA
glu phe arg his asp ser gly tyr glu val his his gln lys leu val phe phe ala glu
1191 /   41                                               1221 /   51
GAT GTG GGT TCA AAC AAA GGT GCA ATC ATT GGA CTC ATG GTG GGC GGT GTT GTC ATA GCG
asp val gly ser asn lys gly ala ile ile gly leu met val gly gly val val ile ala
1251 /   61
TGA
OPA
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Fig. 4

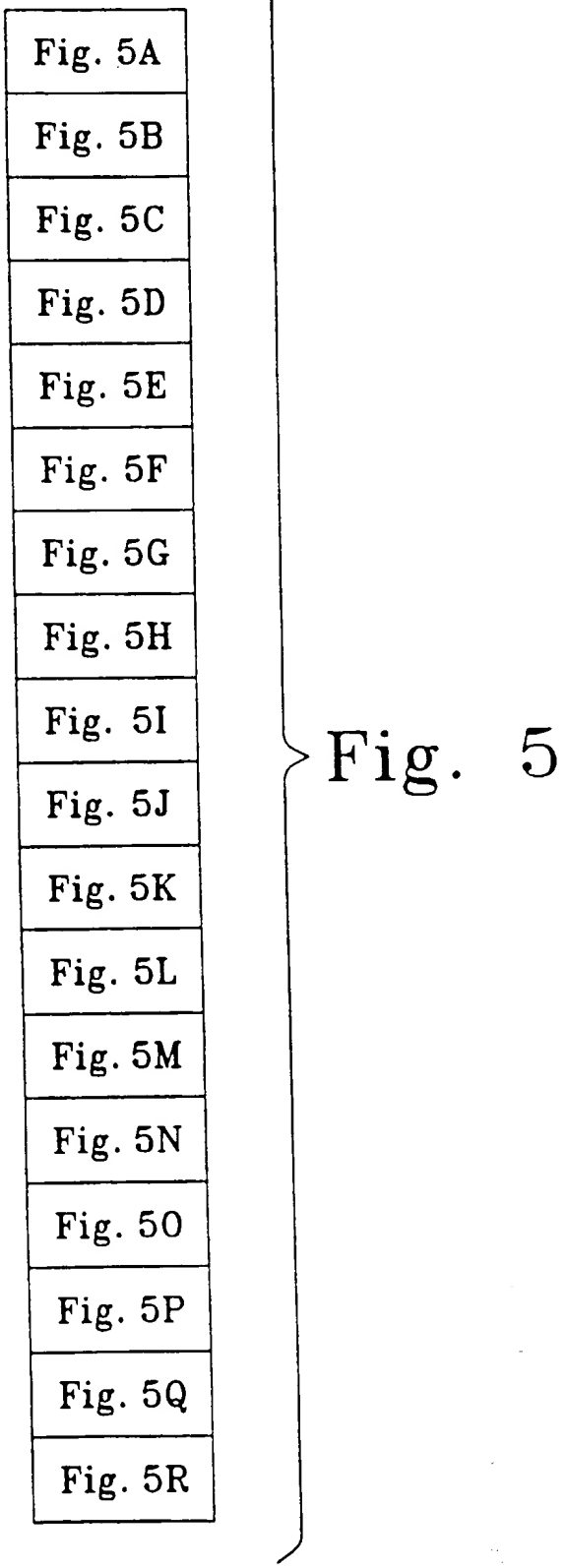
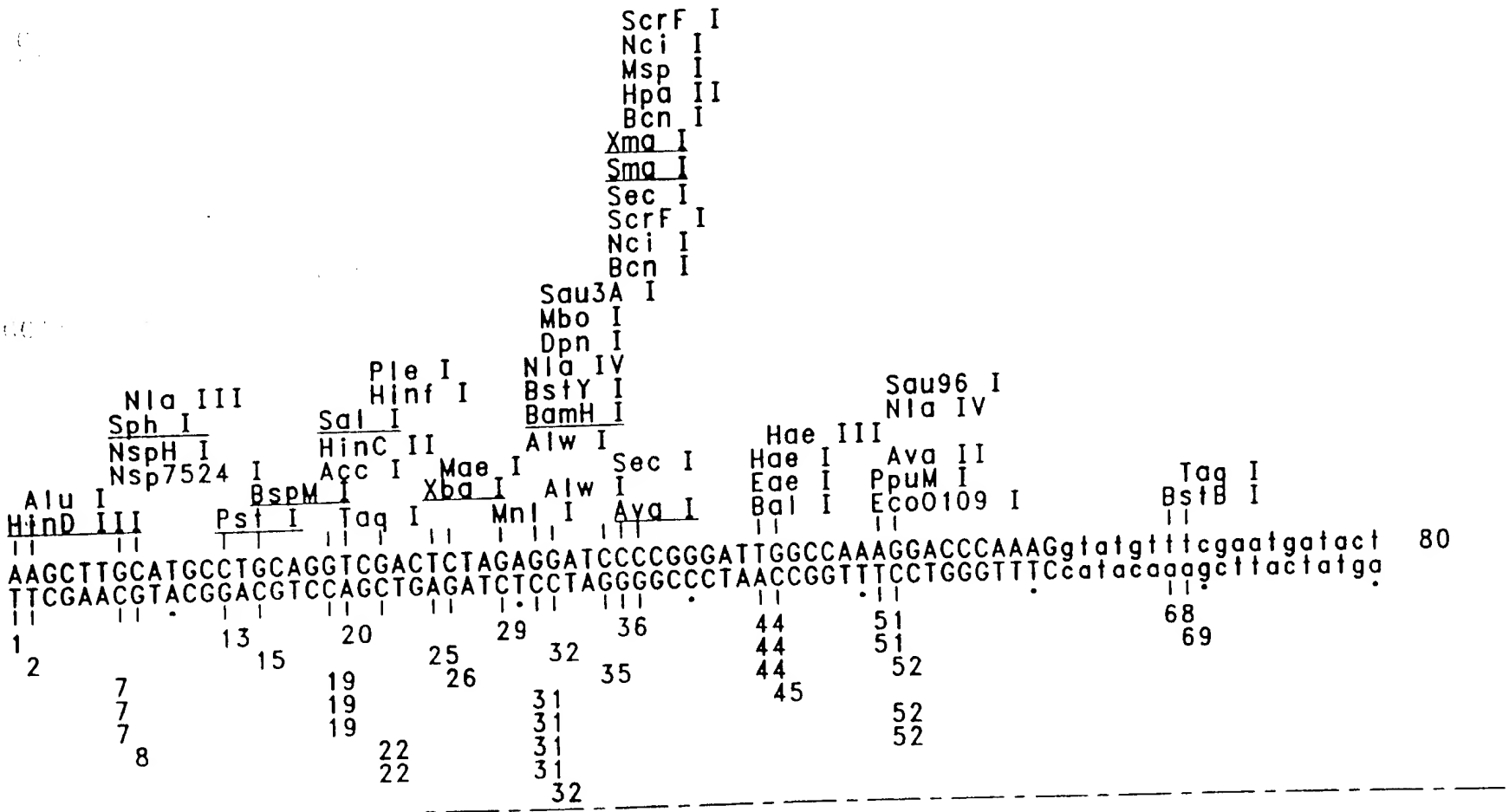


Fig. 5A
Fig. 5B
Fig. 5C
Fig. 5D
Fig. 5E
Fig. 5F
Fig. 5G
Fig. 5H
Fig. 5I
Fig. 5J
Fig. 5K
Fig. 5L
Fig. 5M
Fig. 5N
Fig. 5O
Fig. 5P
Fig. 5Q
Fig. 5R

Fig. 5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Hpa II
Bcn I
Xma I
Sma I
Sec I
ScrF I
Nci I
Bcn I
Sau3A I
Mbo I
Dpn I
Nla IV
BstY I
BamH I
Alw I
Ple I
Hinf I
Nla III
Sph I
NspH I
Nsp7524 I
Sal I
HinC II
Acc I
Mae I
Xba I
Alw I
Sec I
Sau96 I
Nla IV
Hae III
Hae I
Eae I
Bal I
Ava II
PpuM I
Eco0109 I
Taq I
BstB I
Alu I
HinD III
BspM I
Pst I
Taq I
Mnl I
Ava I
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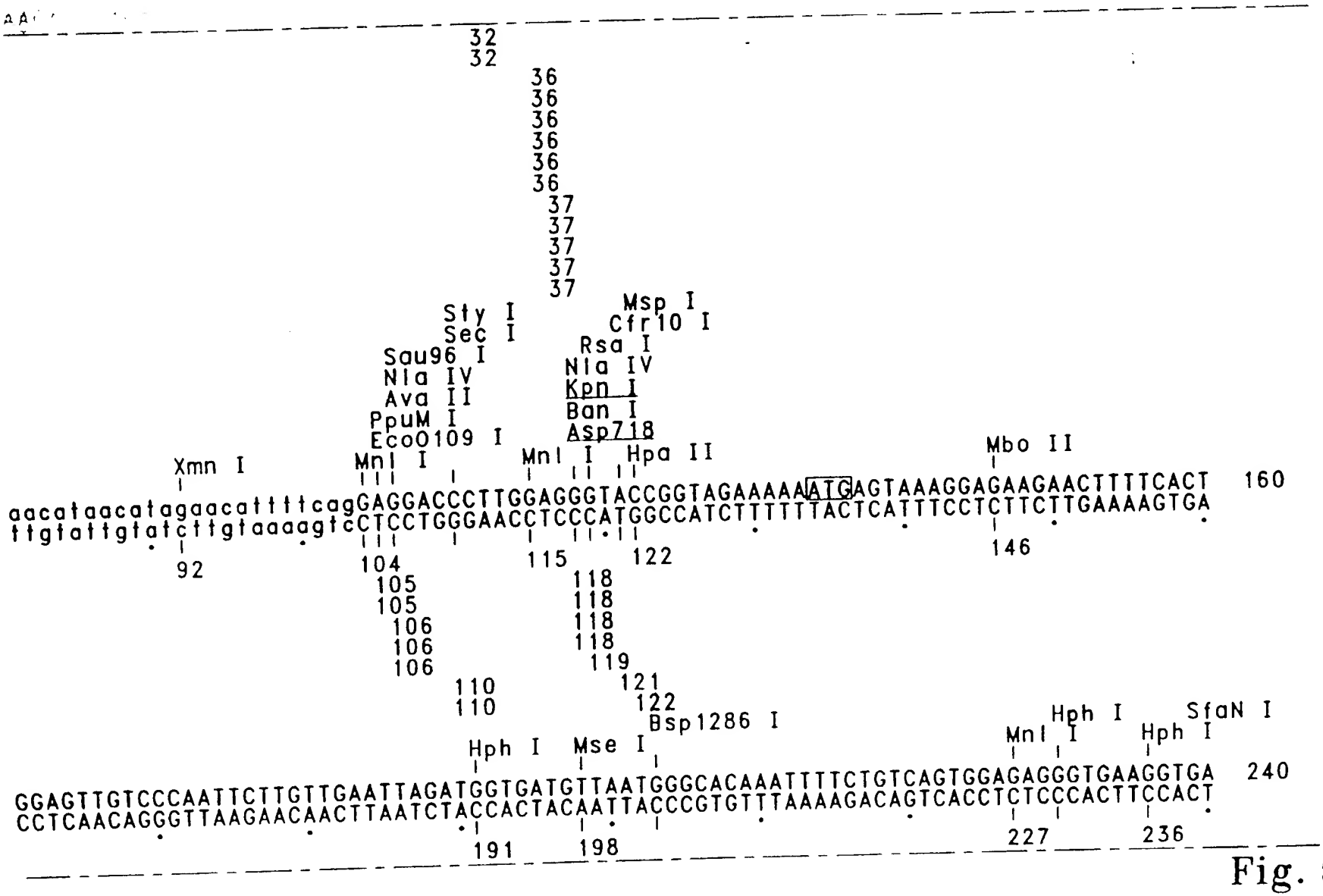
80

Fig. 5A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37
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Sec I
Msp I
Cfr10 I
Rsa I
Sau96 I
Nla IV
Nla IV
Kpn I
Ava II
Ban I
PpuM I
Asp718
Eco0109 I
Xmn I
Mnl I
Mnl I
Hpa II
Mbo II
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92
104
115
122
146
105
105
106
106
106
118
118
118
118
119
110
110
121
122
Bsp1286 I
Hph I
Mse I
Hph I
Mnl I
Hph I
SfaN I
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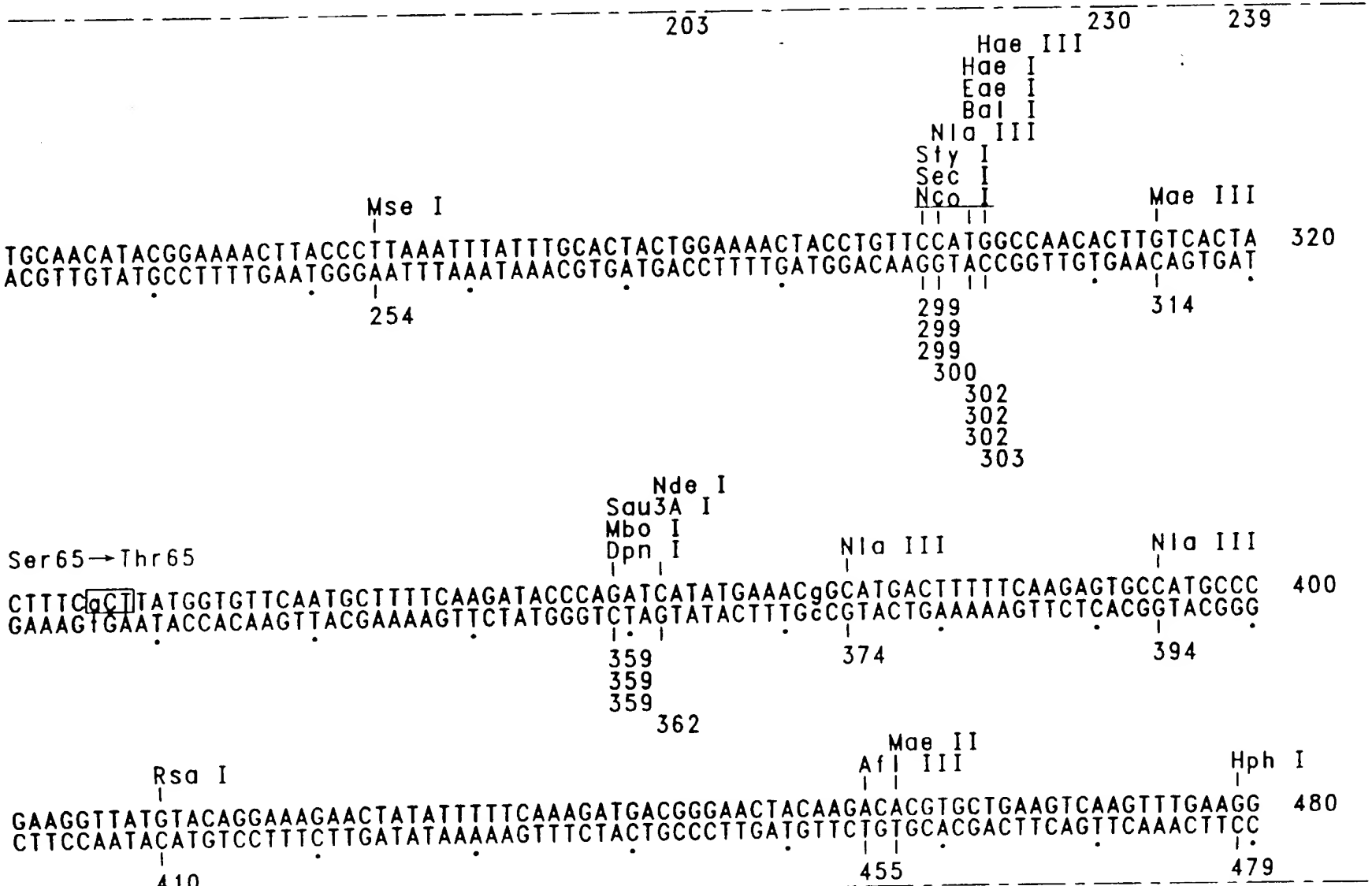
198
227
236

Fig. 5B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230
239
Hae III
Hae I
Eae I
Bal I
Nla III
Sty I
Sec I
Nco I
Mse I
Mae III
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ACGTTGTATGCCTTTTGAATGGGAATTTAAATAAACGTGATGACCTTTTGATGGACAAGGTACCGGTTGTGAACAGTGAT
254
299
299
299
300
302
302
302
303
314
Nde I
Sau3A I
Mbo I
Dpn I
Nla III
Nla III
Ser65→Thr65
CTTTCaCTTATGGTGTTCAATGCTTTTCAAGATACCCAGATCATATGAAACgGCATGACTTTTTCAAGAGTGCCATGCCC 400
GAAAGTGAATACCACAAGTTACGAAAAGTTCTATGGGTCTAGTATACTTTGcCGTACTGAAAAAGTTCTCACGGTACGGG
359
359
359
362
374
394
Mae II
Af I III
Rsa I
Hph I
GAAGGTTATGTACAGGAAAGAACTATATTTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGG 480
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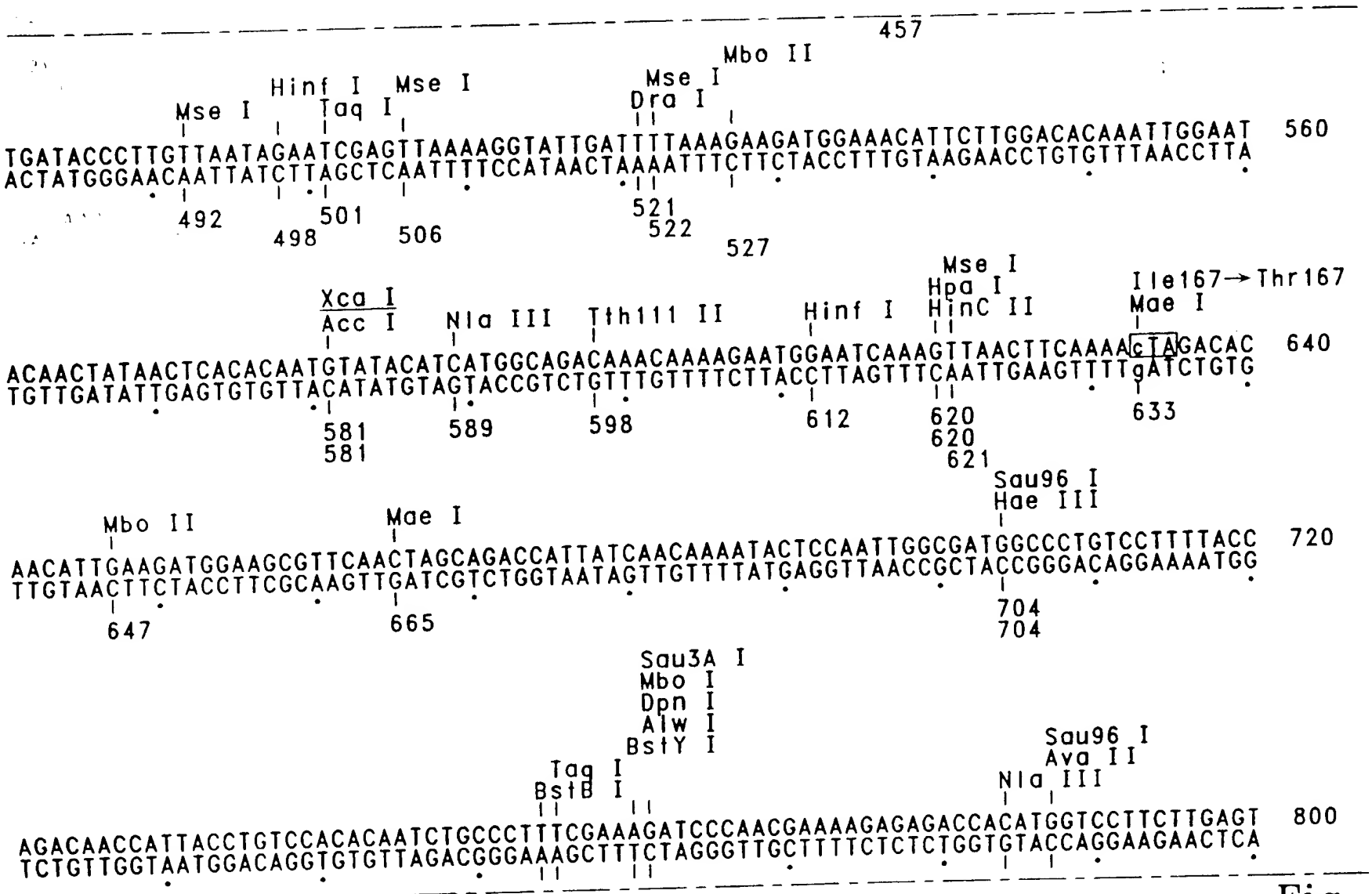
410
455
479

Fig. 5C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Hinf I
Mse I
Mse I
Mse I
Taq I
Dra I
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ACTATGGGAACAATTATCTTAGCTCAATTTTCCATAACTAAAATTTCTTCTACCTTTGTAAGAACCTGTGTTTAACCTTA
492
498
501
506
521
522
527
Mse I
Hpa I
Ile167→Thr167
Xca I
Acc I
Nla III
Tth111 II
Hinf I
HinC II
Mae I
ACAACTATAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAAAAcTAGACAC 640
TGTTGATATTGAGTGTGTTACATATGTAGTACCGTCTGTTTGTTTTCTTACCTTAGTTTCAATTGAAGTTTTgATCTGTG
581
581
589
598
612
620
620
621
633
Sau96 I
Hae III
Mbo II
Mae I
AACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACC 720
TTGTAACTTCTACCTTCGCAAGTTGATCGTCTGGTAATAGTTGTTTTATGAGGTTAACCGCTACCGGGACAGGAAAATGG
647
665
704
704
Sau3A I
Mbo I
Dpn I
Alw I
BstY I
Sau96 I
Ava II
Taq I
BstB I
Nla III
AGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGT 800
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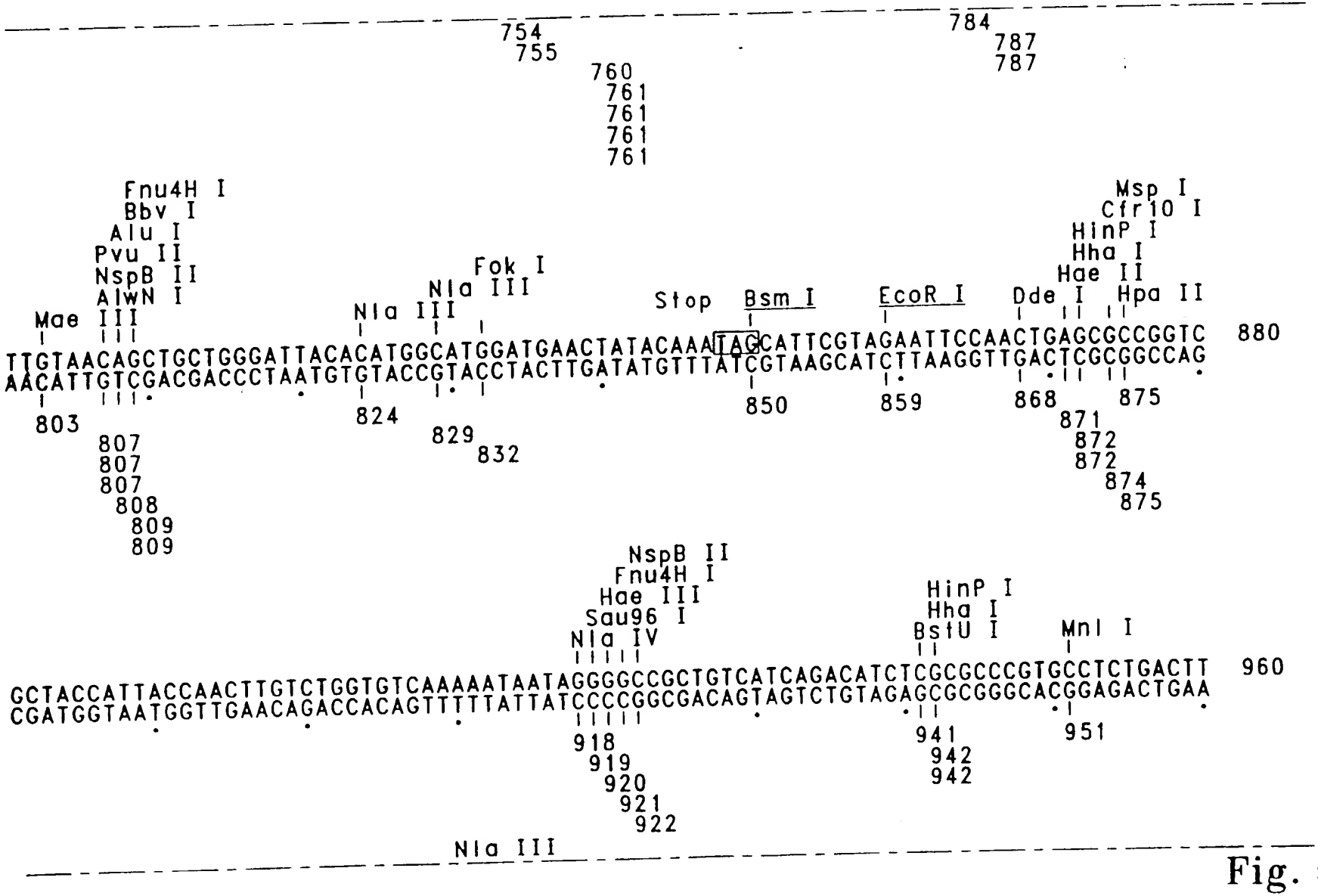
Fig. 5D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761
784
787
787
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Bbv I
Alu I
Pvu II
NspB II
AlwN I
Mae III
Nla III
Fok I
Nla III
Stop
Bsm I
EcoR I
Dde I
Hpa II
Msp I
Cfr10 I
HinP I
Hha I
Hae II
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AACATTGTCGACGACCCTAATGTGTACCGTACCTACTTGATATGTTTATCGTAAGCATCTTAAGGTTGACTCGCGGCCAG
803
807
807
807
808
809
809
824
829
832
850
859
868
871
872
872
874
875
875
NspB II
Fnu4H I
Hae III
Sau96 I
Nla IV
HinP I
Hha I
BstU I
MnI I
GCTACCATTACCAACTTGTCTGGTGTCAAAAATAATAGGGGCCGCTGTCATCAGACATCTCGCGCCCGTGCCTCTGACTT 960
CGATGGTAATGGTTGAACAGACCACAGTTTTTATTATCCCCGGCGACAGTAGTCTGTAGAGCGCGGGCACGGAGACTGAA
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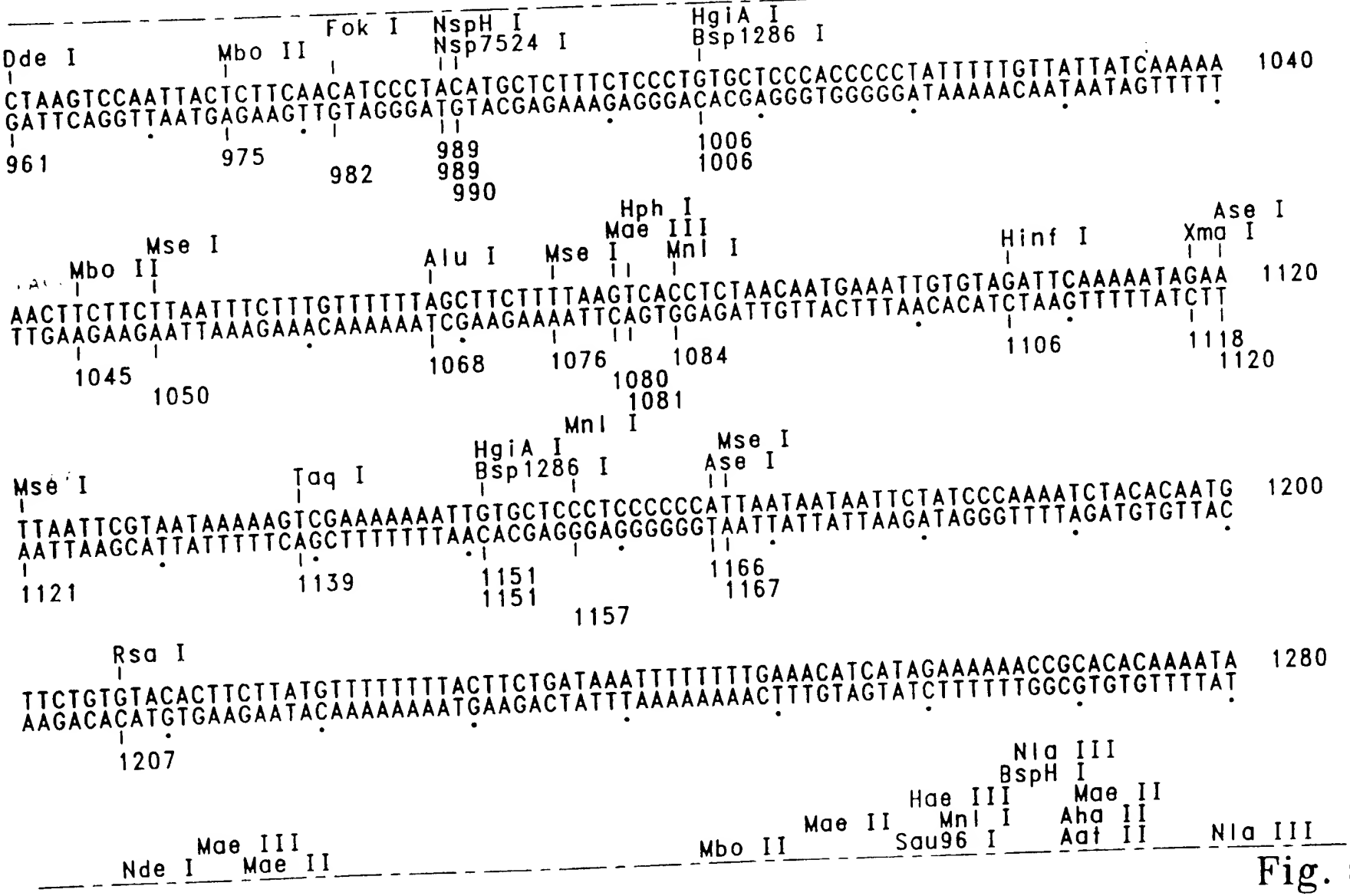
919
920
921
922
941
942
942
951
Nla III

Fig. 5E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Nsp7524 I
HgiA I
Bsp1286 I
Dde I
Mbo II
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GATTCAGGTTAATGAGAAGTTGTAGGGATGTACGAGAAAGAGGGACACGAGGGTGGGGGATAAAAACAATAATAGTTTTT
961
975
982
989
989
990
1006
1006
Hph I
Mae III
Ase I
Mse I
Mbo II
Alu I
Mse I
Mnl I
Hinf I
Xma I
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TTGAAGAAGAATTAAAGAAACAAAAAATCGAAGAAAATTCAGTGGAGATTGTTACTTTAACACATCTAAGTTTTTATCTT
1045
1050
1068
1076
1080
1081
1084
1106
1118
1120
Mnl I
HgiA I
Bsp1286 I
Mse I
Ase I
Mse I
Taq I
TTAATTCGTAATAAAAAGTCGAAAAAAATTGTGCTCCCTCCCCCCATTAATAATAATTCTATCCCAAAATCTACACAATG 1200
AATTAAGCATTATTTTTCAGCTTTTTTTAACACGAGGGAGGGGGGTAATTATTATTAAGATAGGGTTTTAGATGTGTTAC
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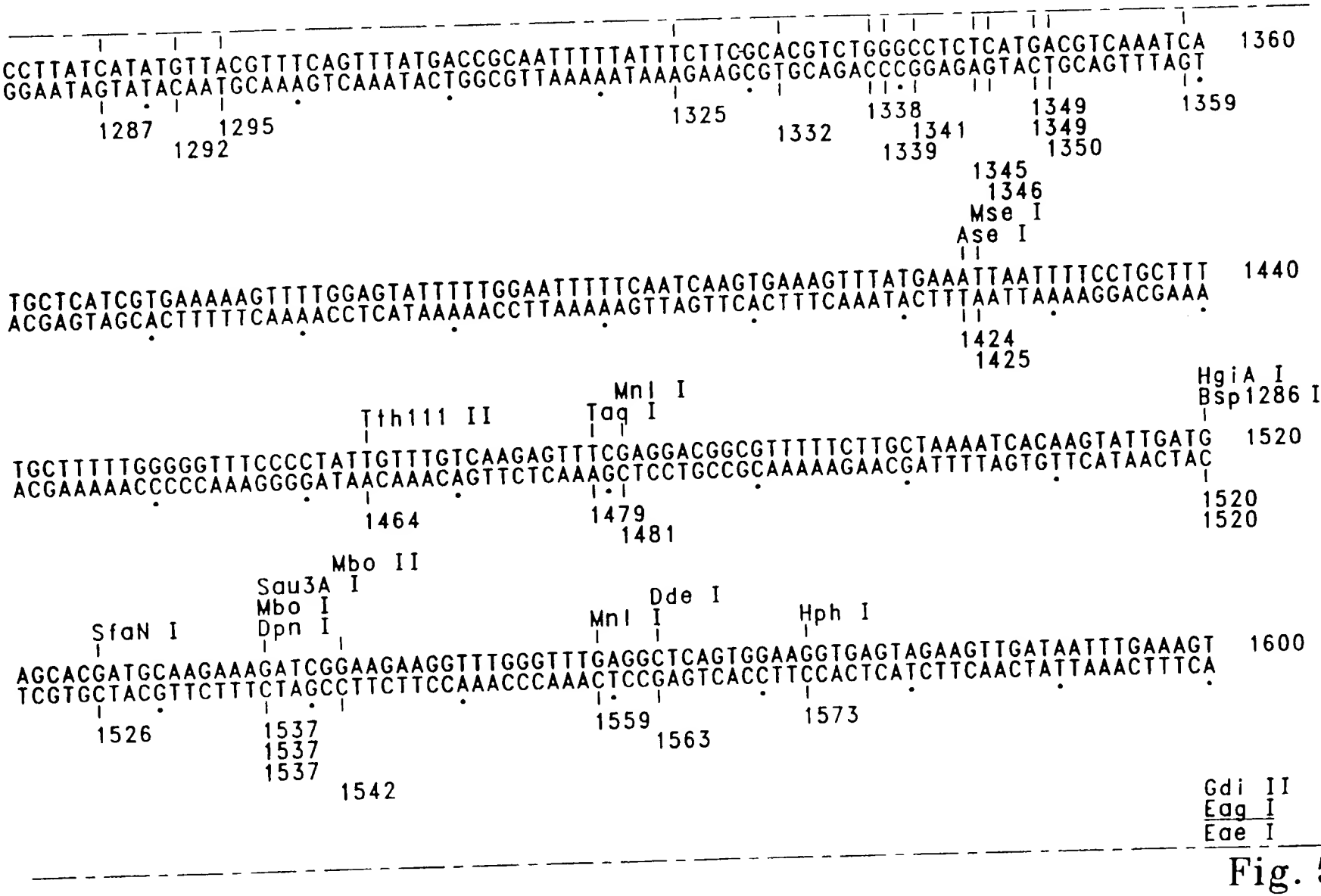
1139
1151
1151
1157
1166
1167
Rsa I
TTCTGTGTACACTTCTTATGTTTTTTTTACTTCTGATAAATTTTTTTTTGAAACATCATAGAAAAAACCGCACACAAAATA 1280
AAGACACATGTGAAGAATACAAAAAAAAATGAAGACTATTTAAAAAAAACTTTGTAGTATCTTTTTTGGCGTGTGTTTTAT
1207
Nla III
BspH I
Hae III
Mae II
Mae II
Mnl I
Aha II
Mae III
Mbo II
Sau96 I
Aat II
Nla III
Nde I
Mae II

Fig. 5F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1287 1295 1325 1338 1349 1359
1292 1332 1341 1349
1339 1350
1345
1346
Mse I
Ase I
TGCTCATCGTGAAAAAGTTTTGGAGTATTTTTGGAATTTTTCAATCAAGTGAAAGTTTATGAAATTAATTTTCCTGCTTT 1440
ACGAGTAGCACTTTTTCAAAACCTCATAAAAACCTTAAAAAGTTAGTTCACTTTCAAATACTTTAATTAAAAGGACGAAA
1424
1425
HgiA I
Bsp1286 I
Mnl I
Tth111 II
Taq I
TGCTTTTTGGGGGTTTCCCCTATTGTTTGTCAAGAGTTTCGAGGACGGCGTTTTTTCTTGCTAAAATCACAAGTATTGATG 1520
ACGAAAAACCCCCAAAGGGGATAACAAACAGTTCTCAAAGCTCCTGCCGCAAAAAGAACGATTTTAGTGTTCATAACTAC
1520
1464 1479 1520
1481
Mbo II
Sau3A I
Mbo I
Dpn I
Dde I
Mnl I
Hph I
SfaN I
AGCACGATGCAAGAAAGATCGGAAGAAGGTTTGGGTTTGAGGCTCAGTGGAAGGTGAGTAGAAGTTGATAATTTGAAAGT 1600
TCGTGCTACGTTCTTTCTAGCCTTCTTCCAAACCCAAACTCCGAGTCACCTTCCACTCATCTTCAACTATTAAACTTTCA
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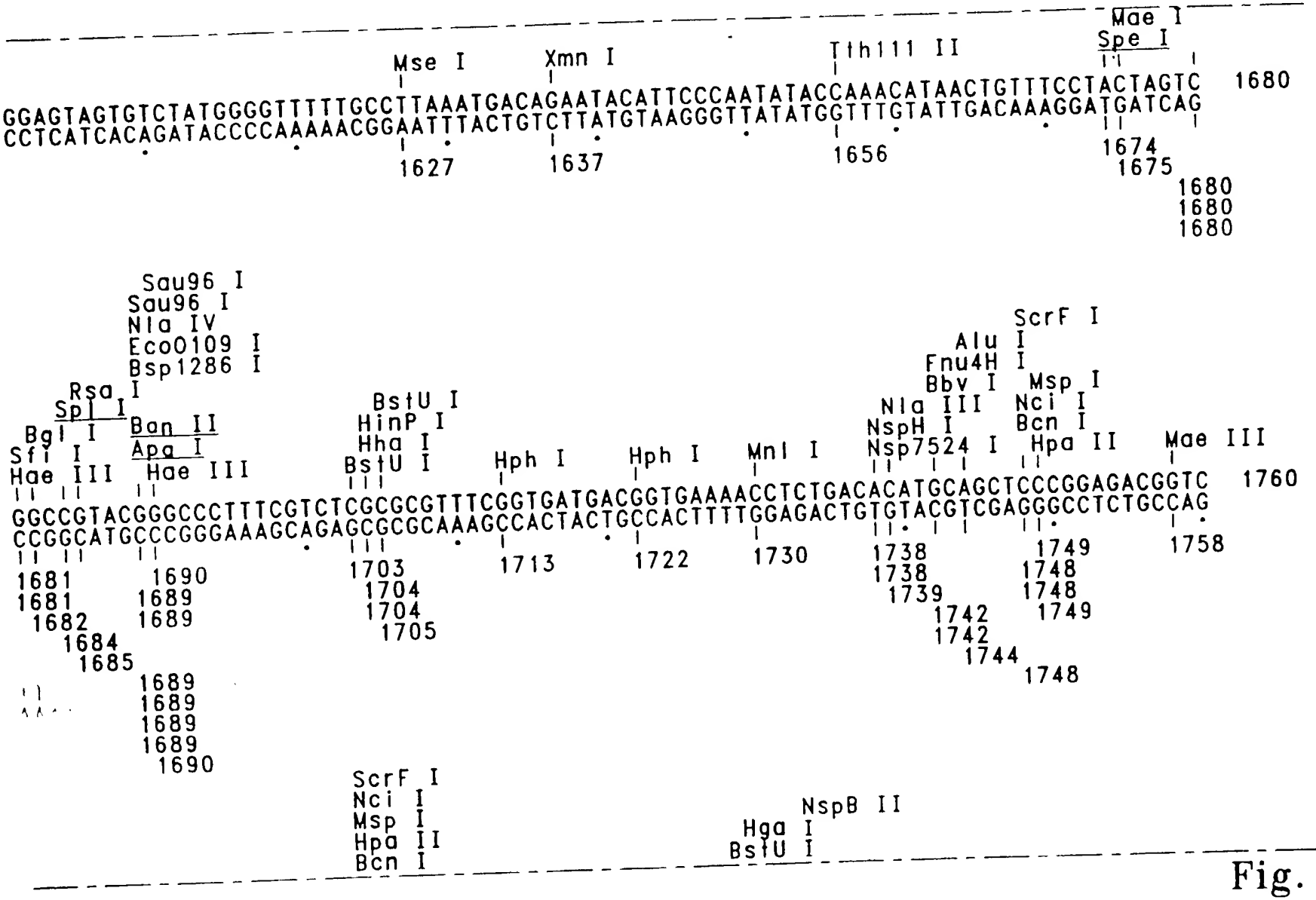
1537
1537
1542
Gdi II
Eag I
Eae I

Fig. 5G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Spe I
Mse I
Xmn I
Tth111 II
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1627
1637
1656
1674
1675
1680
1680
1680
Sau96 I
Sau96 I
Nla IV
Eco0109 I
Bsp1286 I
Rsa I
Sp I I
Bgl I
Ban II
Apa I
Sfi I
Hae III
Hae III
BstU I
HinP I
Hha I
BstU I
Hph I
Hph I
Mnl I
ScrF I
Alu I
Fnu4H I
Bbv I
Msp I
Nla III
Nci I
NspH I
Bcn I
Nsp7524 I
Hpa II
Mae III
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1749
1748
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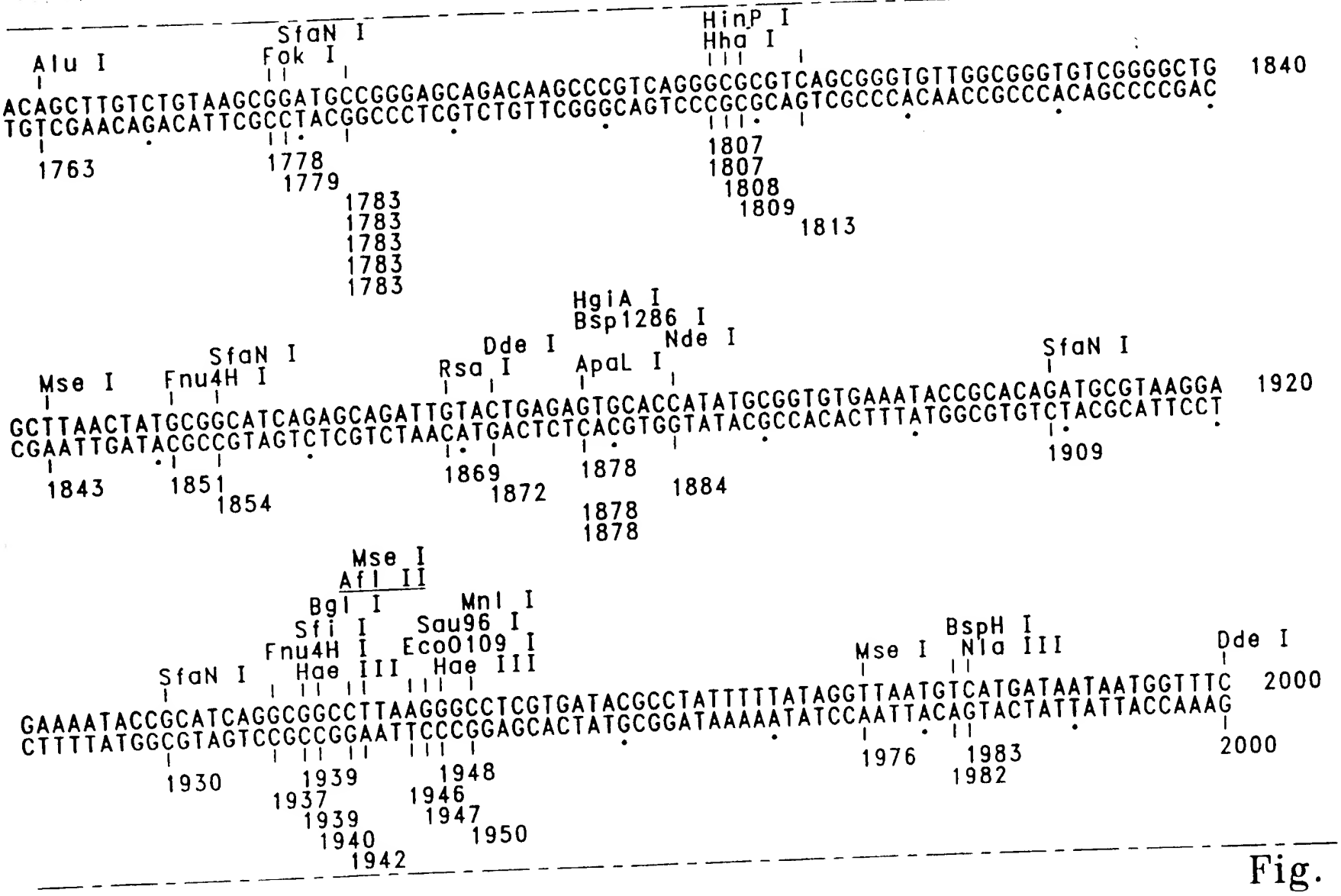
ScrF I
Nci I
Msp I
Hpa II
Bcn I
NspB II
Hga I
BstU I

Fig. 5H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1763
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1783
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1783
1783
1783
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1807
1808
1809
1813
HgiA I
Bsp1286 I
Dde I
Nde I
SfaN I
Mse I
Fnu4H I
Rsa I
ApaL I
SfaN I
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1843
1851
1854
1869
1872
1878
1878
1878
1884
1909
Mse I
Afl II
Bgl I
Mnl I
Sfi I
Sau96 I
Fnu4H I
Eco0109 I
SfaN I
Hae III
Hae III
BspH I
Mse I
Nla III
Dde I
GAAAATACCGCATCAGGCGGCCTTAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTC 2000
CTTTTATGGCGTAGTCCGCCGGAATTCCCGGAGCACTATGCGGATAAAAATATCCAATTACAGTACTATTATTACCAAAG
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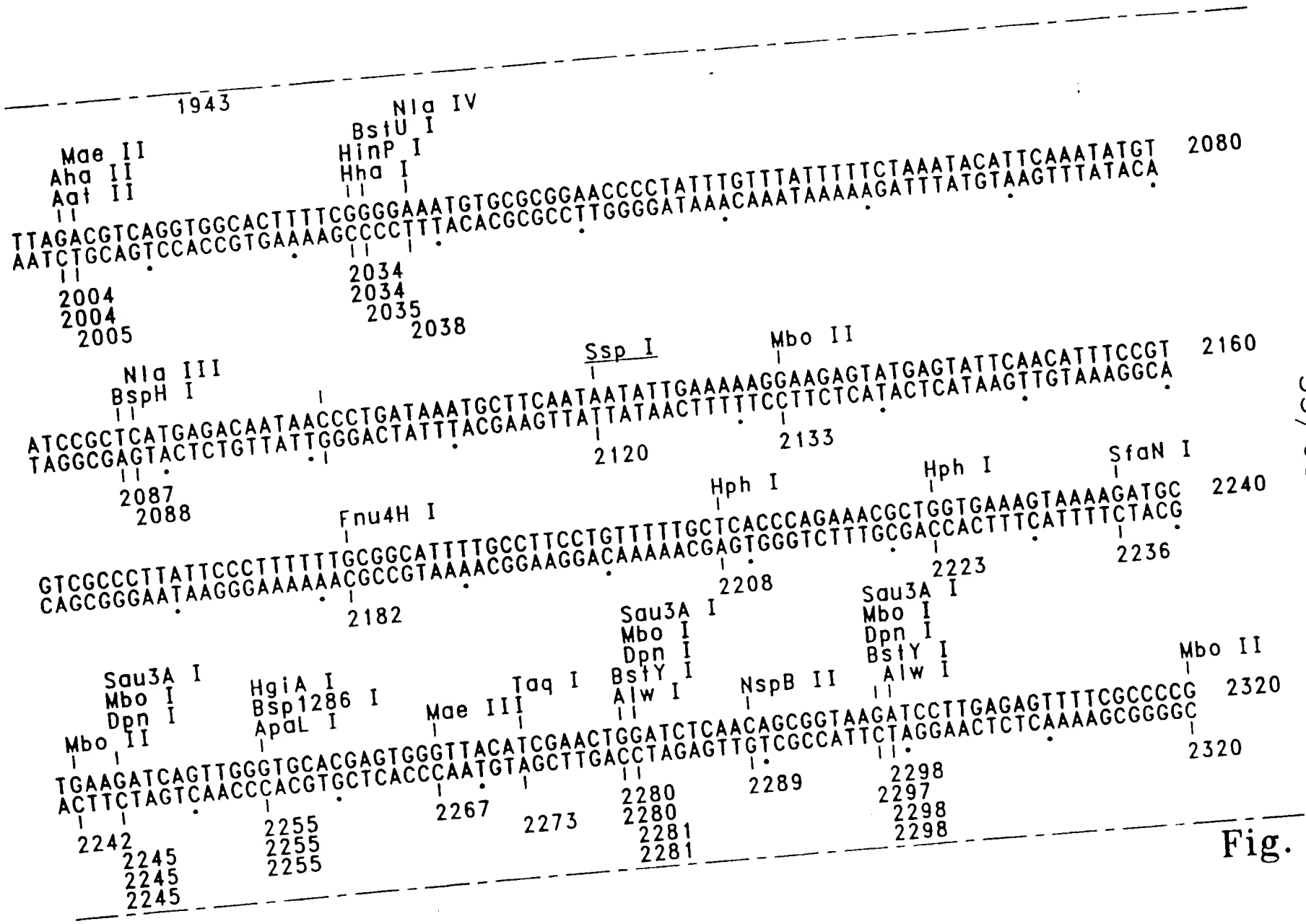
1982
1983
2000

Fig. 5I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BstU I
Mae II
HinP I
Aha II
Hha I
Aat II
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2004
2034
2004
2034
2005
2035
2038
Ssp I
Mbo II
Nla III
BspH I
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TAGGCGAGTACTCTGTTATTGGGACTATTTACGAAGTTATTATAACTTTTTCCTTCTCATACTCATAAGTTGTAAAGGCA
2120
2133
2087
2088
Hph I
Hph I
SfaN I
Fnu4H I
GTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGC 2240
CAGCGGGAATAAGGGAAAAAACGCCGTAAAACGGAAGGACAAAAACGAGTGGGTCTTTGCGACCACTTTCATTTTCTACG
2182
2208
2223
2236
Sau3A I
Mbo I
Dpn I
Sau3A I
Mbo I
Dpn I
BstY I
Alw I
Mbo II
Sau3A I
Mbo I
Dpn I
HgiA I
Bsp1286 I
ApaL I
Taq I
BstY I
Alw I
NspB II
Mae III
Mbo II
TGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCG 2320
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2320
2242
2255
2267
2273
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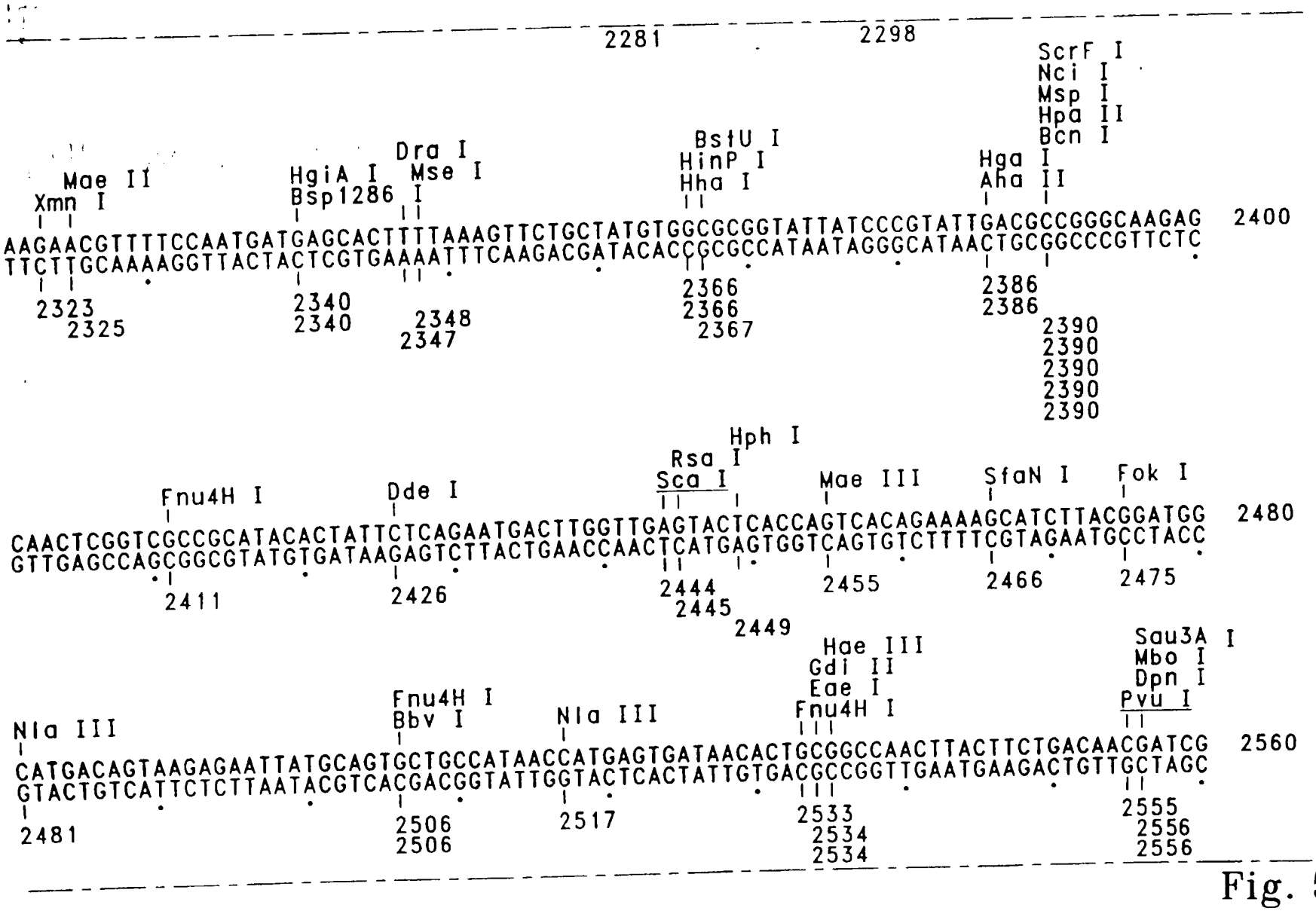
2298
2245
2281

Fig. 5J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Msp I
Hpa II
Bcn I
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Aha II
Dra I
Mse I
HgiA I
Bsp1286 I
Mae II
Xmn I
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2367
2386
2386
2390
2390
2390
2390
2390
Hph I
Rsa I
Sca I
Fnu4H I
Dde I
Mae III
SfaN I
Fok I
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2466
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Gdi II
Eae I
Fnu4H I
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Mbo I
Dpn I
Pvu I
Nla III
Fnu4H I
Bbv I
Nla III
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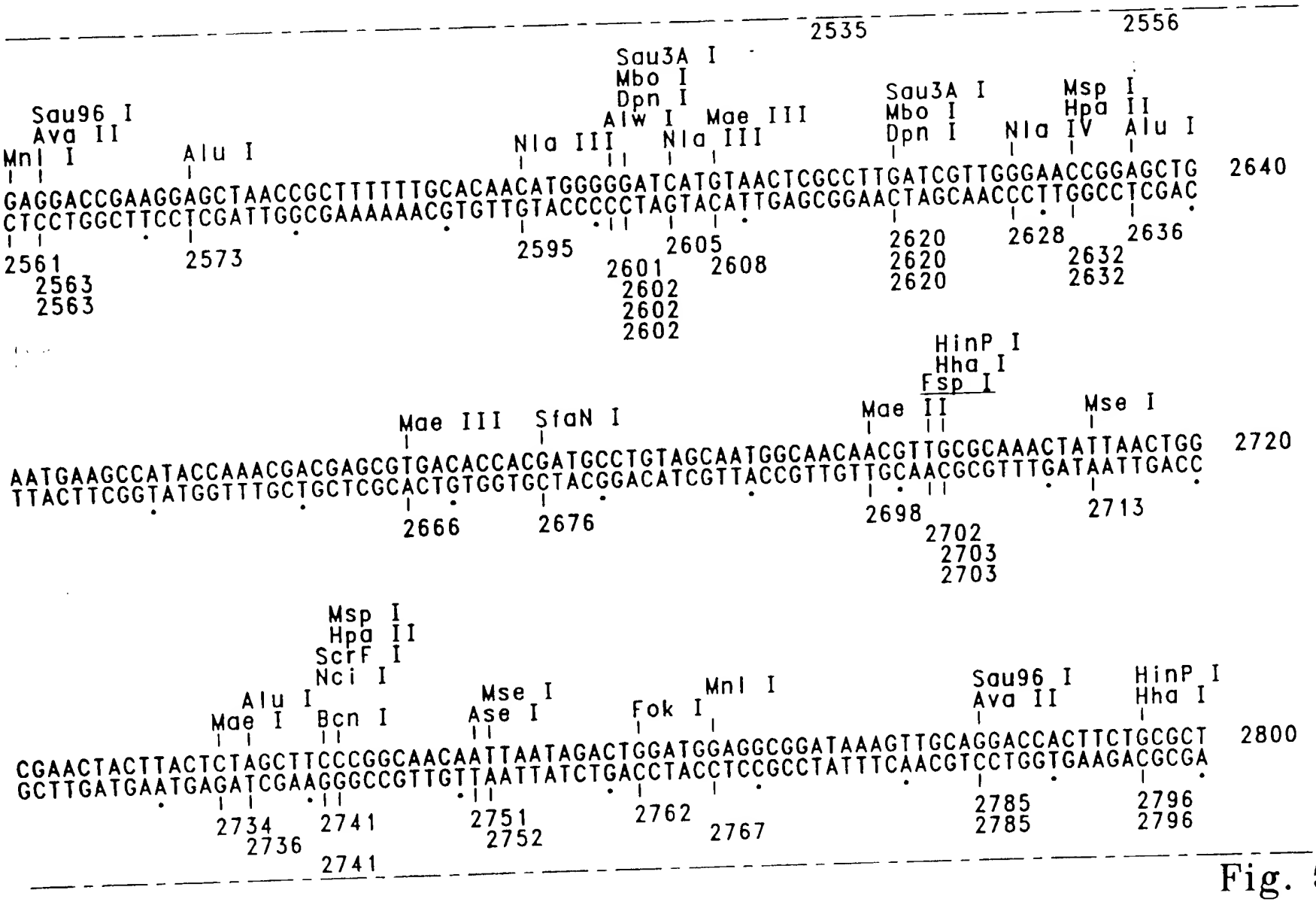
Fig. 5K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Alu I
Nla III
Alw I
Mae III
Sau3A I
Mbo I
Dpn I
Msp I
Hpa II
Nla IV
Alu I
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CTCCTGGCTTCCTCGATTGGCGAAAAAACGTGTTGTACCCCCTAGTACATTGAGCGGAACTAGCAACCCTTGGCCTCGAC
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2602
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HinP I
Hha I
Fsp I
Mae III
SfaN I
Mae II
Mse I
AATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGG 2720
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2703
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Msp I
Hpa II
ScrF I
Nci I
Alu I
Mae I
Bcn I
Mse I
Ase I
Fok I
Mnl I
Sau96 I
Ava II
HinP I
Hha I
CGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCT 2800
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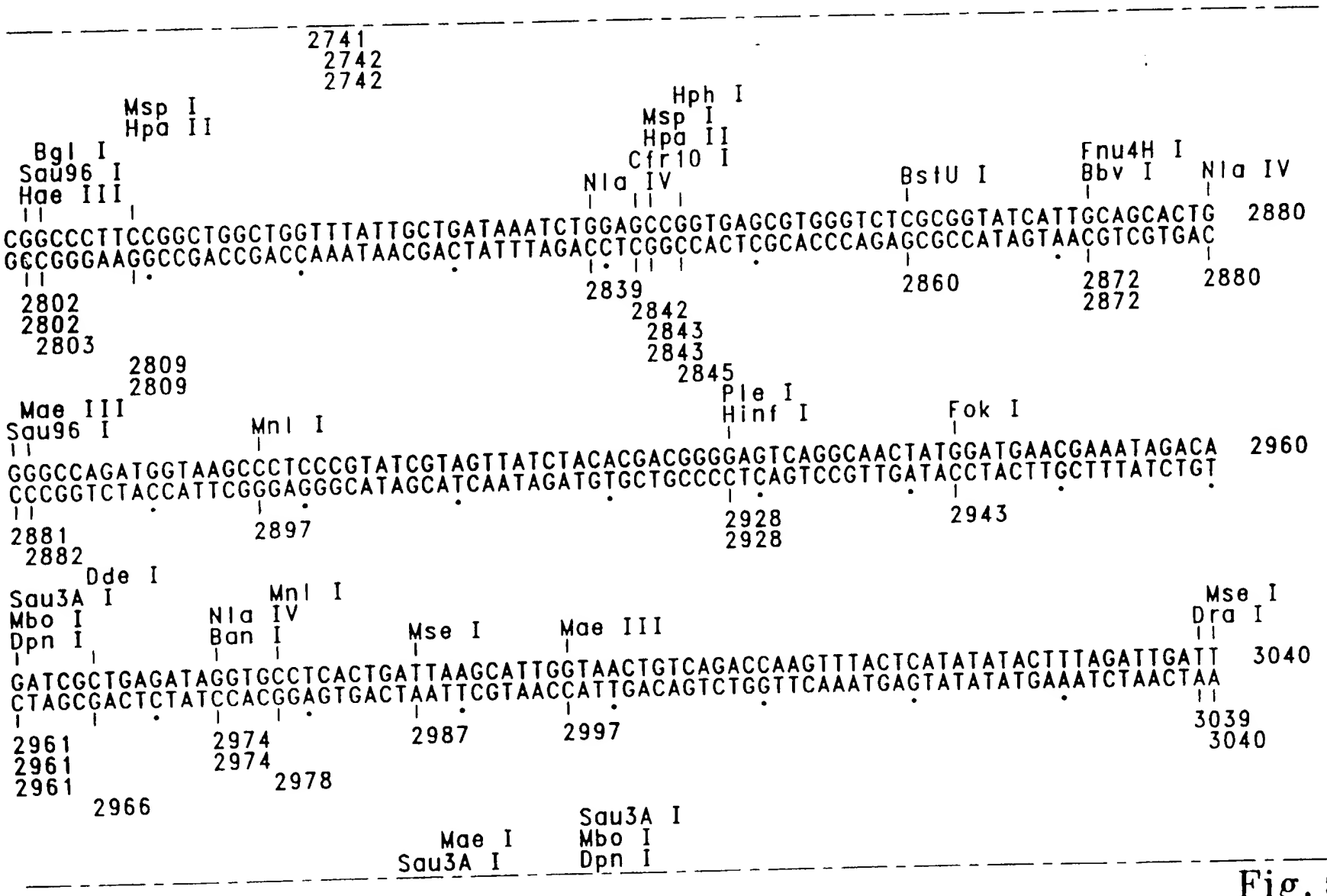
Fig. 5L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BstU I
Fnu4H I
Bbv I
Nla IV
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Mae III
Sau96 I
Mnl I
Ple I
Hinf I
Fok I
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Sau3A I
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Dpn I
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Nla IV
Ban I
Mse I
Mae III
Mse I
Dra I
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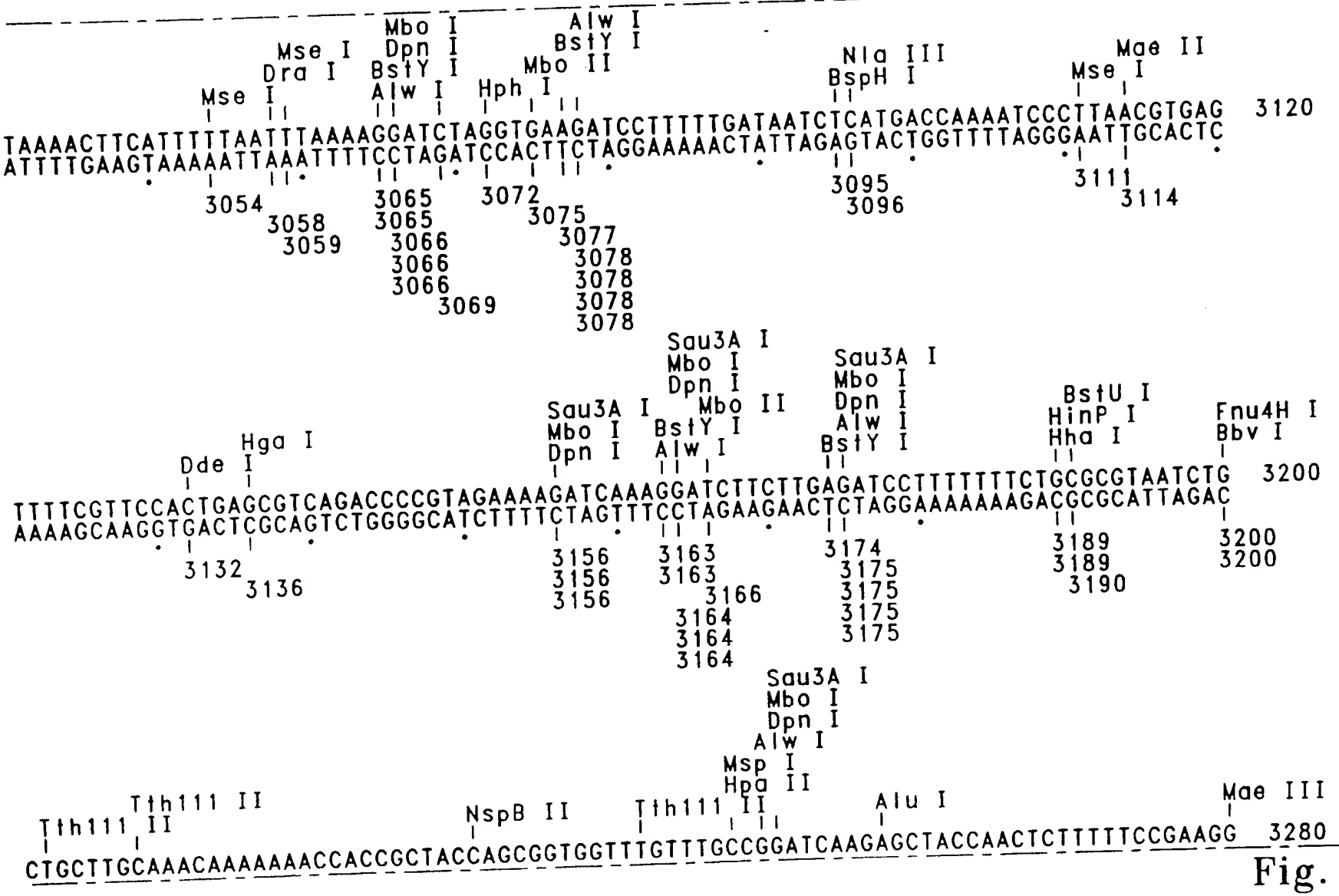
Mbo I
Dpn I

Fig. 5M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Alw I
Hph I
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3114
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Mbo I
Dpn I
Sau3A I
Mbo I
Dpn I
Alw I
BstY I
Mbo II
Sau3A I
Mbo I
Dpn I
BstY I
Alw I
BstU I
HinP I
Hha I
Fnu4H I
Bbv I
Hga I
Dde I
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3200
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Mbo I
Dpn I
Alw I
Msp I
Hpa II
Tth111 II
Tth111 II
NspB II
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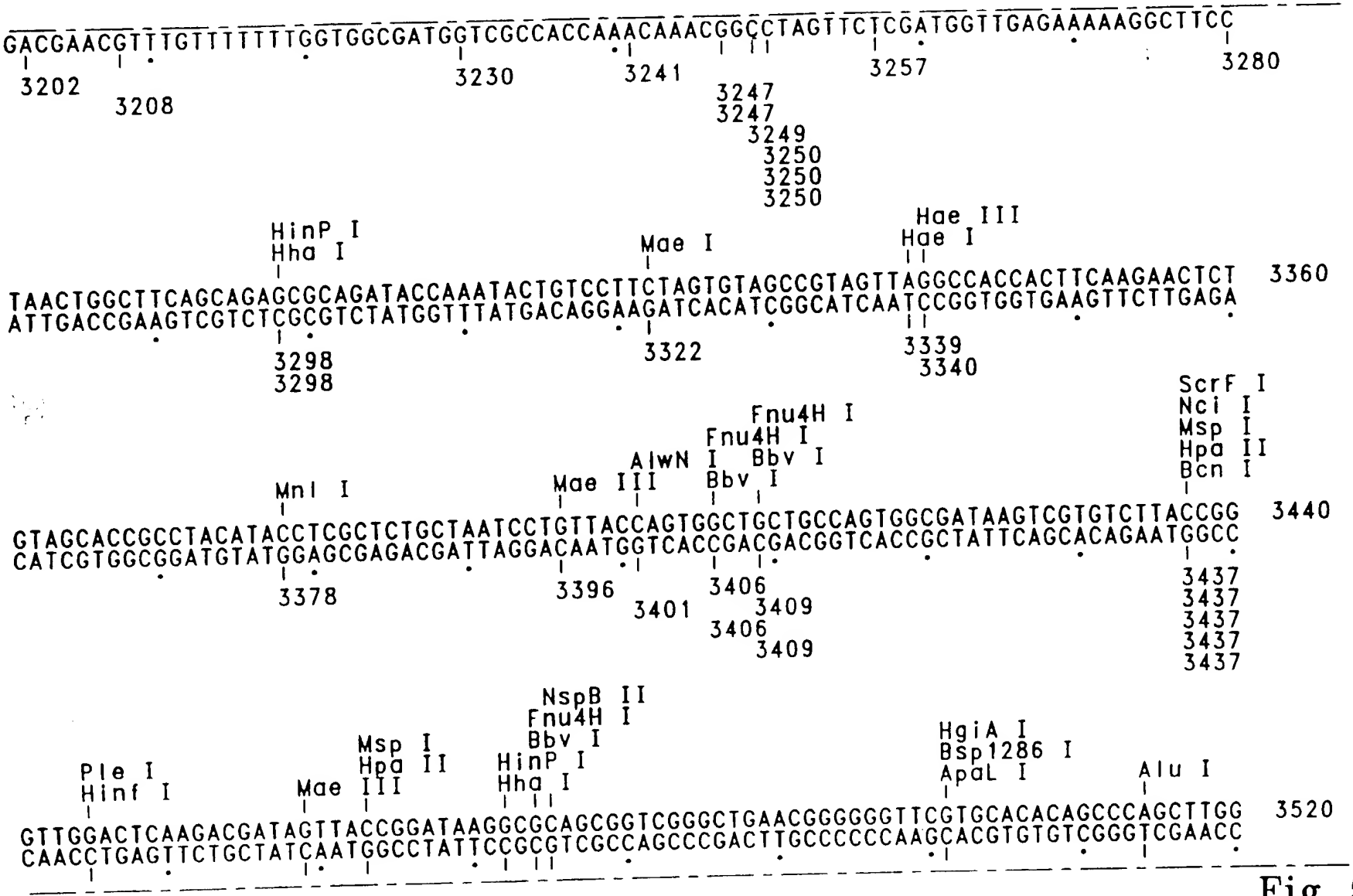
Fig. 5N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HinP I
Hha I
Mae I
Hae III
Hae I
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Nci I
Msp I
Hpa II
Bcn I
Fnu4H I
Fnu4H I
AlwN I
Bbv I
Mnl I
Mae III
Bbv I
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Fnu4H I
Bbv I
Msp I
Hpa II
HinP I
Hha I
Ple I
Hinf I
Mae III
HgiA I
Bsp1286 I
ApaL I
Alu I
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Fig. 50

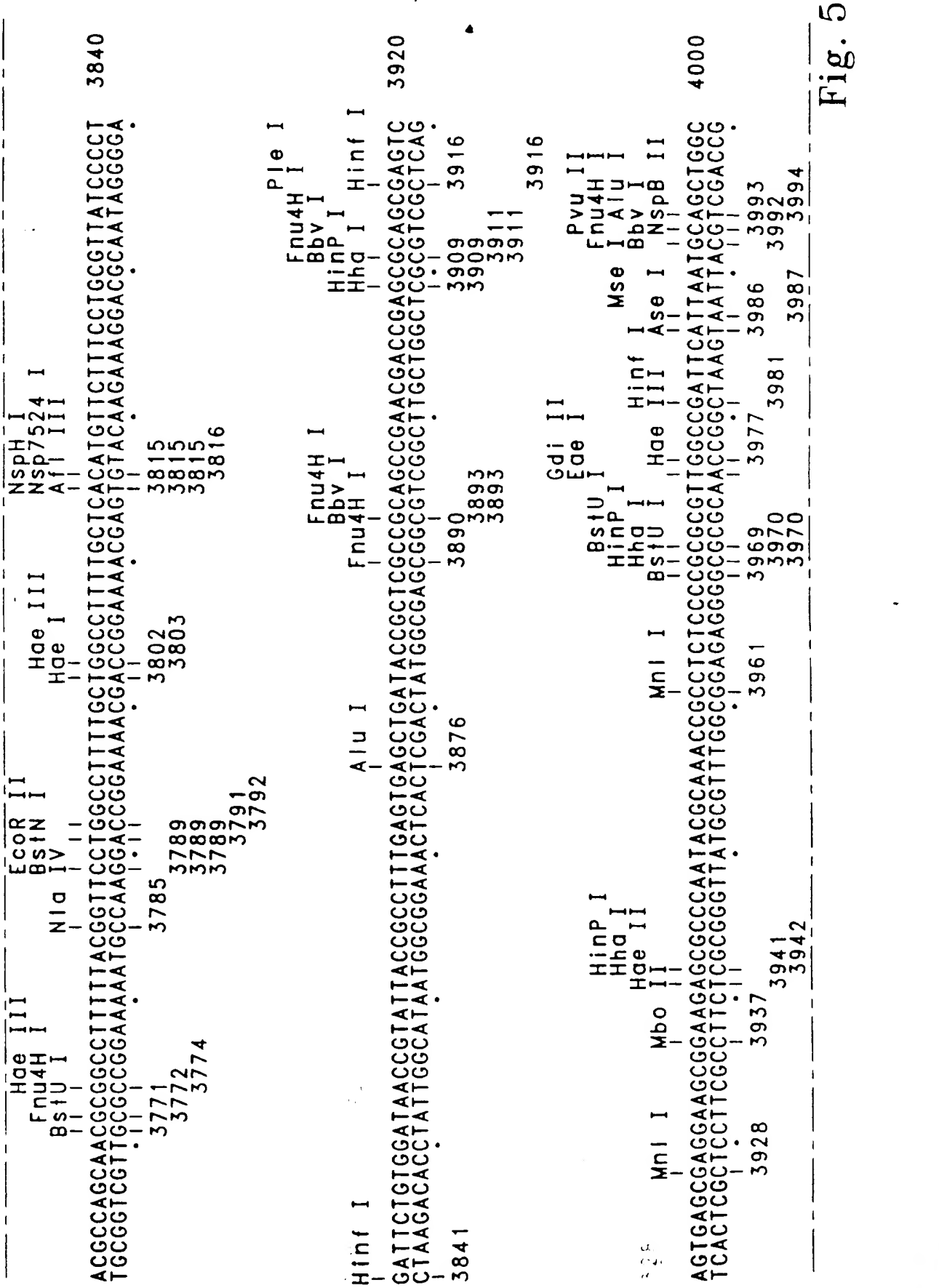

Fig. 5Q

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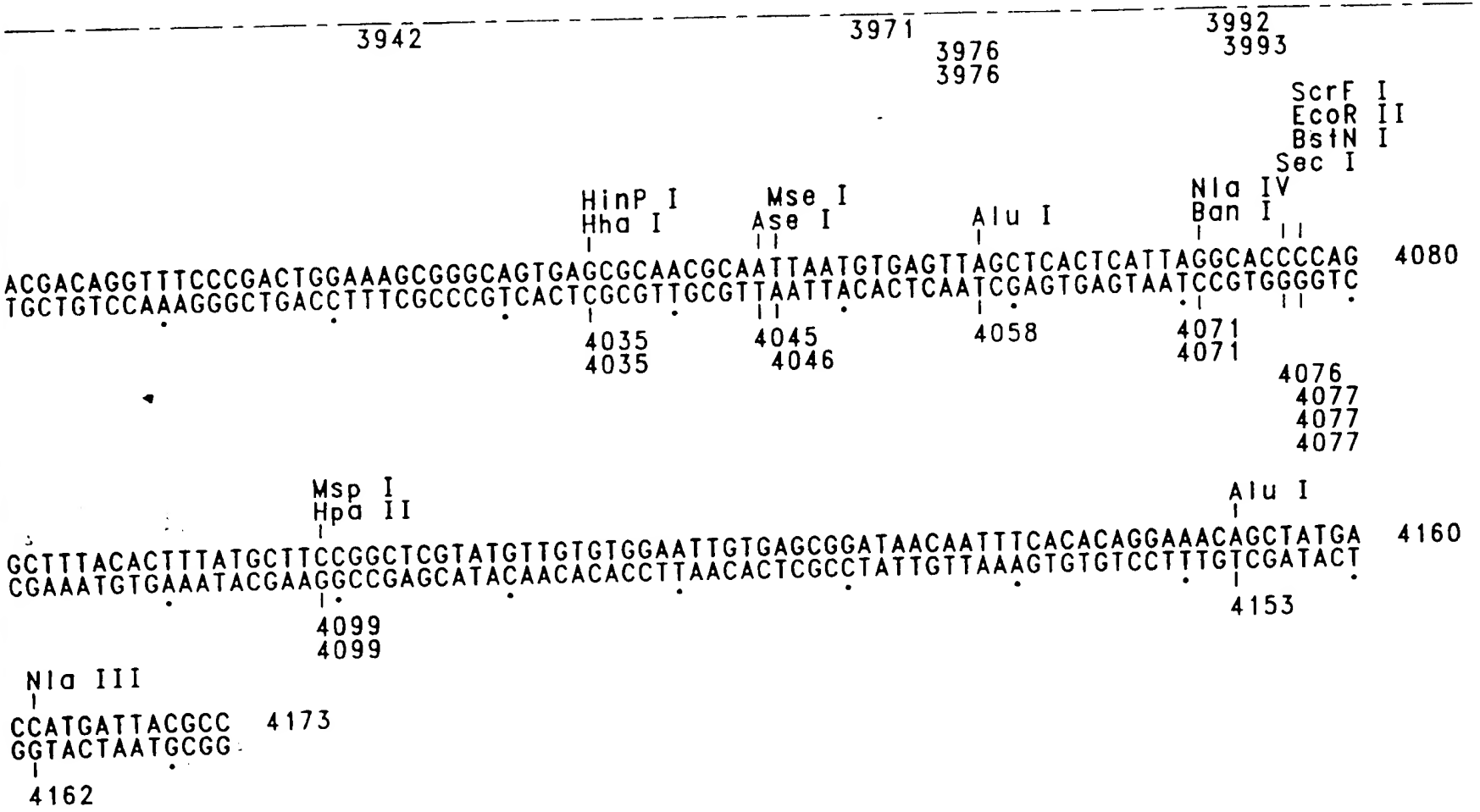
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Sec I
HinP I
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Mse I
Ase I
Alu I
Nla IV
Ban I
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Hpa II
Alu I
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Nla III
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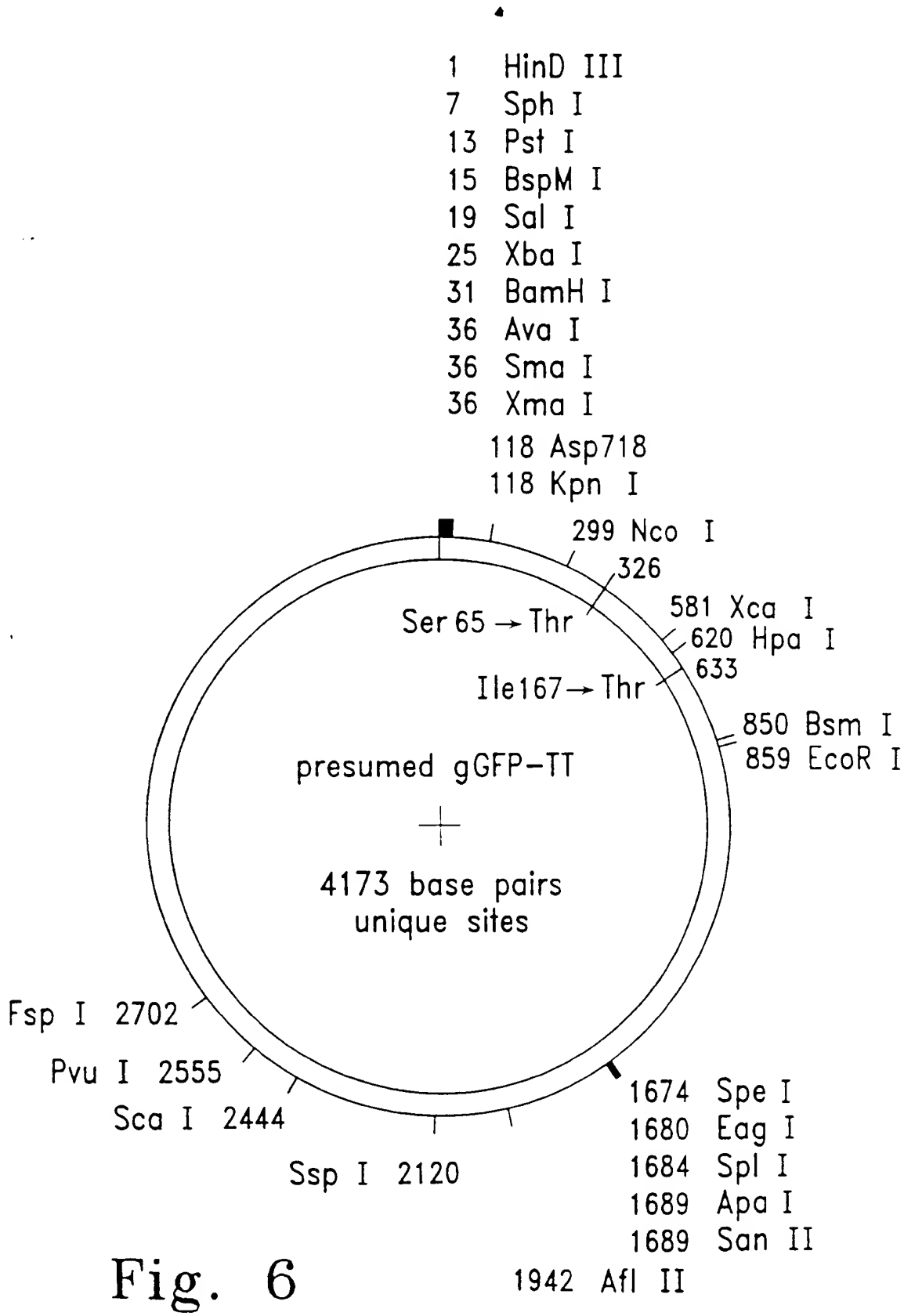
Fig. 5R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7 Sph I
13 Pst I
15 BspM I
19 Sal I
25 Xba I
31 BamH I
36 Ava I
36 Sma I
36 Xma I
118 Asp718
118 Kpn I
299 Nco I
326
581 Xca I
620 Hpa I
633
850 Bsm I
859 EcoR I
Ser 65 → Thr
Ile167 → Thr
presumed gGFP-TT
4173 base pairs
unique sites
Fsp I 2702
Pvu I 2555
Sca I 2444
Ssp I 2120
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1684 Spl I
1689 Apa I
1689 San II
1942 Afl II

Fig. 6

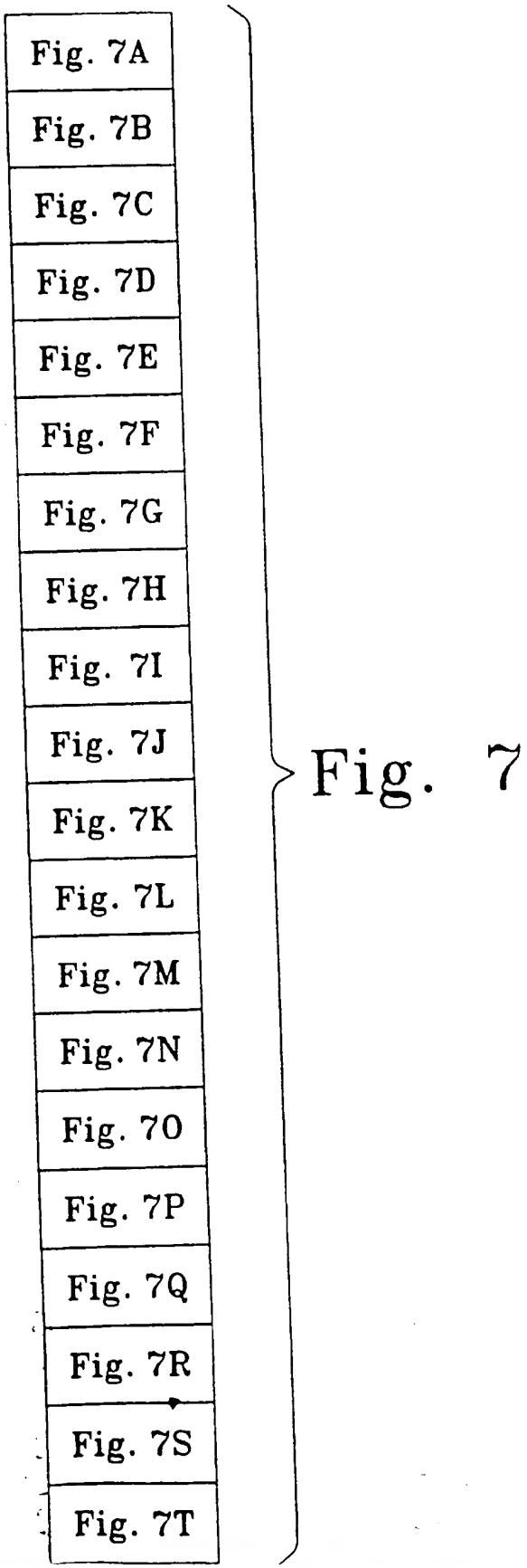
Fig. 7A
Fig. 7B
Fig. 7C
Fig. 7D
Fig. 7E
Fig. 7F
Fig. 7G
Fig. 7H
Fig. 7I
Fig. 7J
Fig. 7K
Fig. 7L
Fig. 7M
Fig. 7N
Fig. 7O
Fig. 7P
Fig. 7Q
Fig. 7R
Fig. 7S
Fig. 7T

Fig. 7

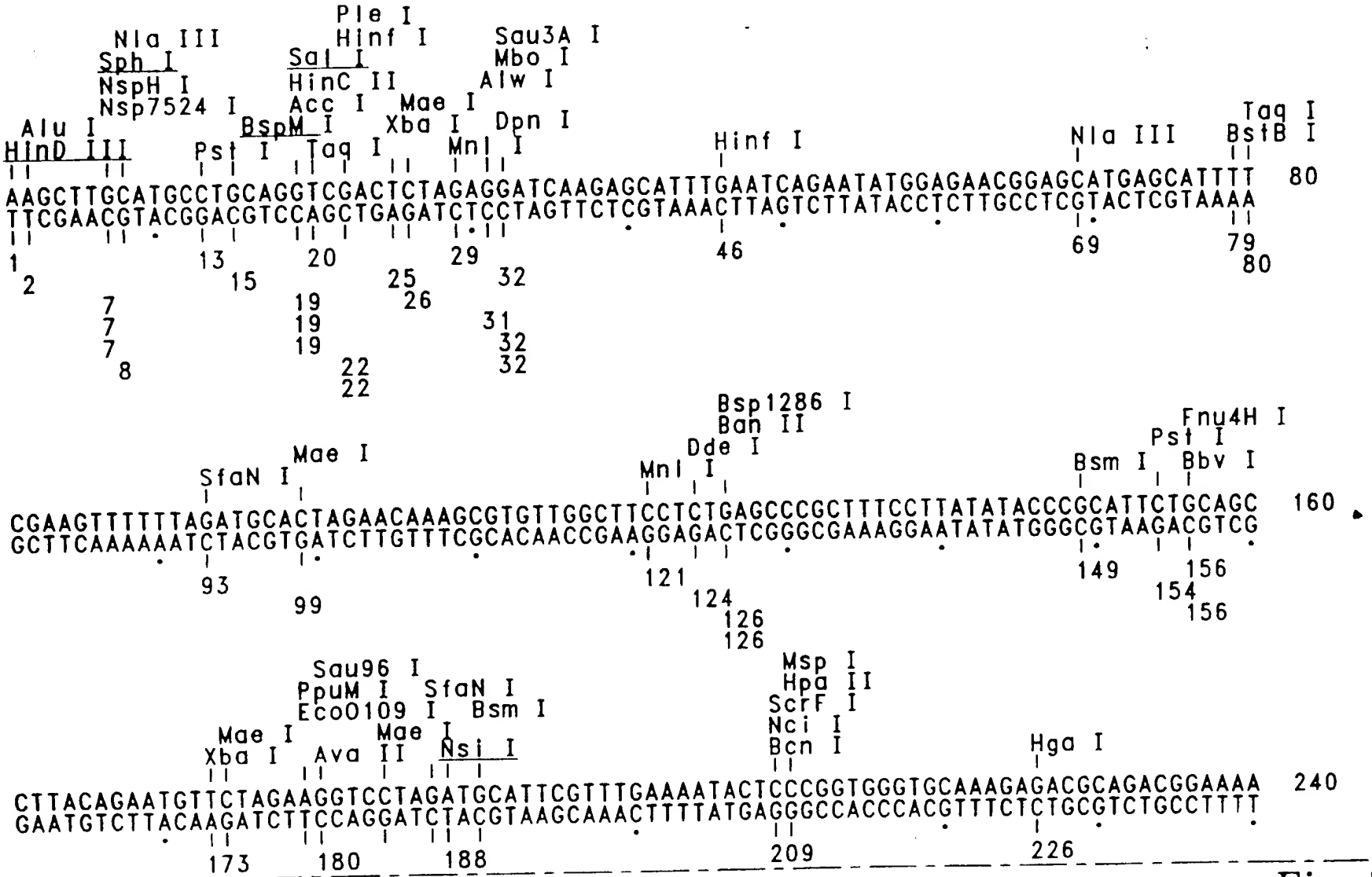

Fig. 7A

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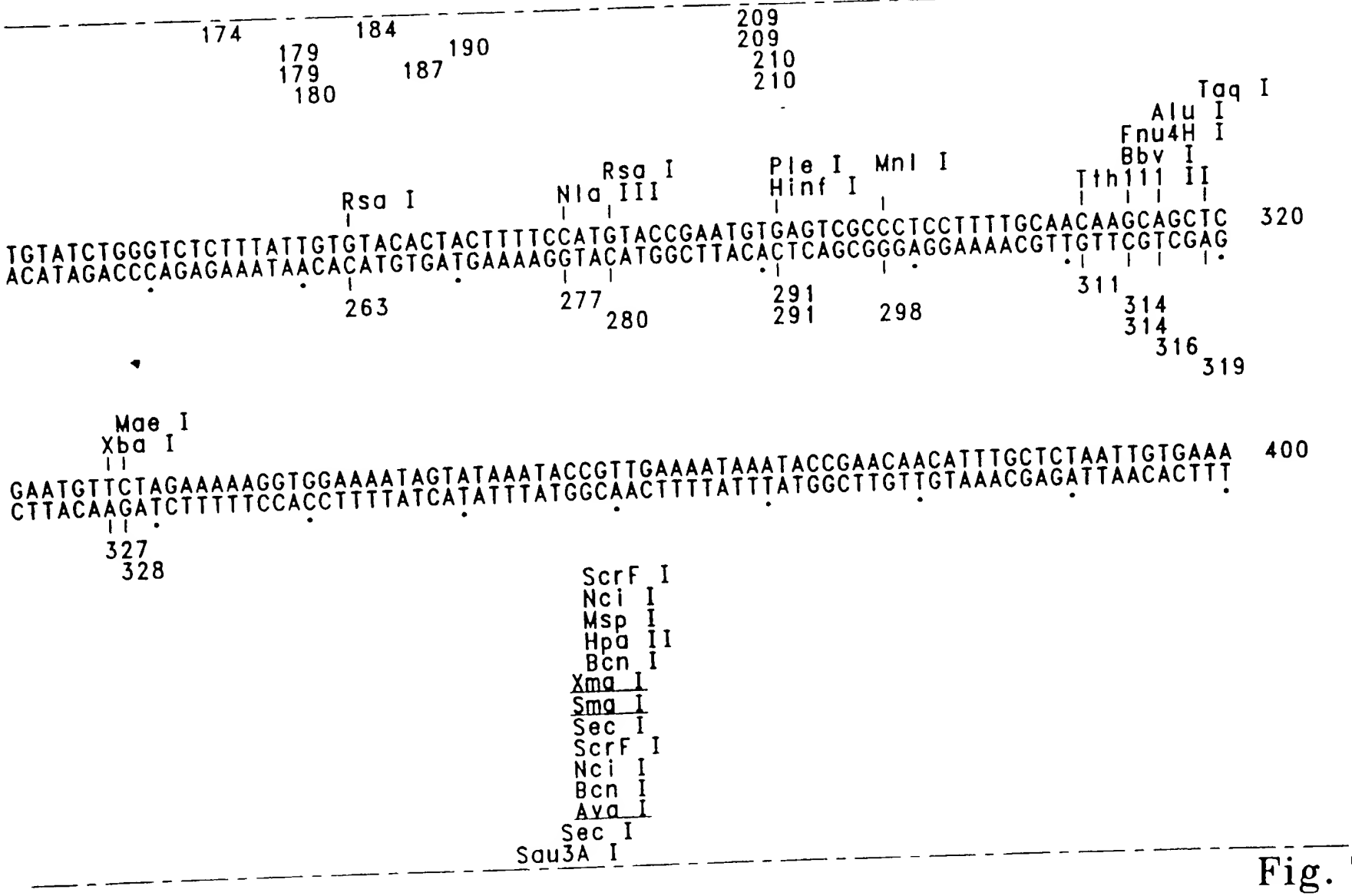
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Alu I
Fnu4H I
Bbv I
Tth111 II
Rsa I
Rsa I
NIa III
Ple I
Hinf I
Mnl I
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ACATAGACCCAGAGAAATAACACATGTGATGAAAAGGTACATGGCTTACACTCAGCGGGAGGAAAACGTTGTTCGTCGAG
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277
280
291
291
298
311
314
314
316
319
Mae I
Xba I
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ScrF I
Nci I
Msp I
Hpa II
Bcn I
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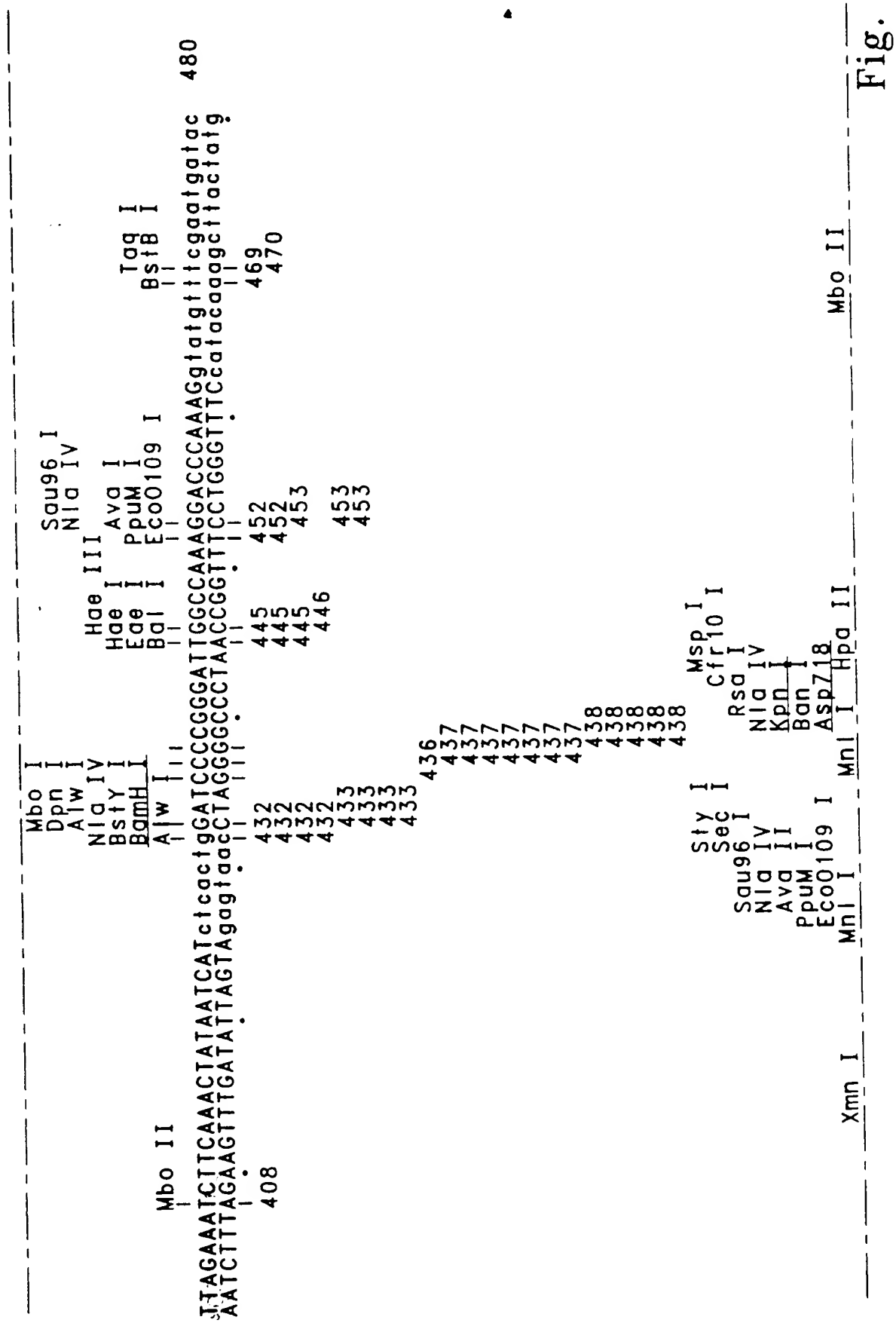
Sma I
Sec I
ScrF I
Nci I
Bcn I
Ava I
Sec I
Sau3A I

Fig. 7B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Nla IV
BstY I
BamH I
Mbo II
Sau96 I
Nla IV
Hae III
Ava I
Hae I
PpuM I
Eae I
EcoO109 I
Bal I
Taq I
BstB I
Mbo II
Xmn I
Mnl I
Msp I
Cfr10 I
Rsa I
Nla IV
Kpn I
Ban I
AspZ18
Hpa II
Sty I
Sec I
Sau96 I
Nla IV
Ava II
PpuM I
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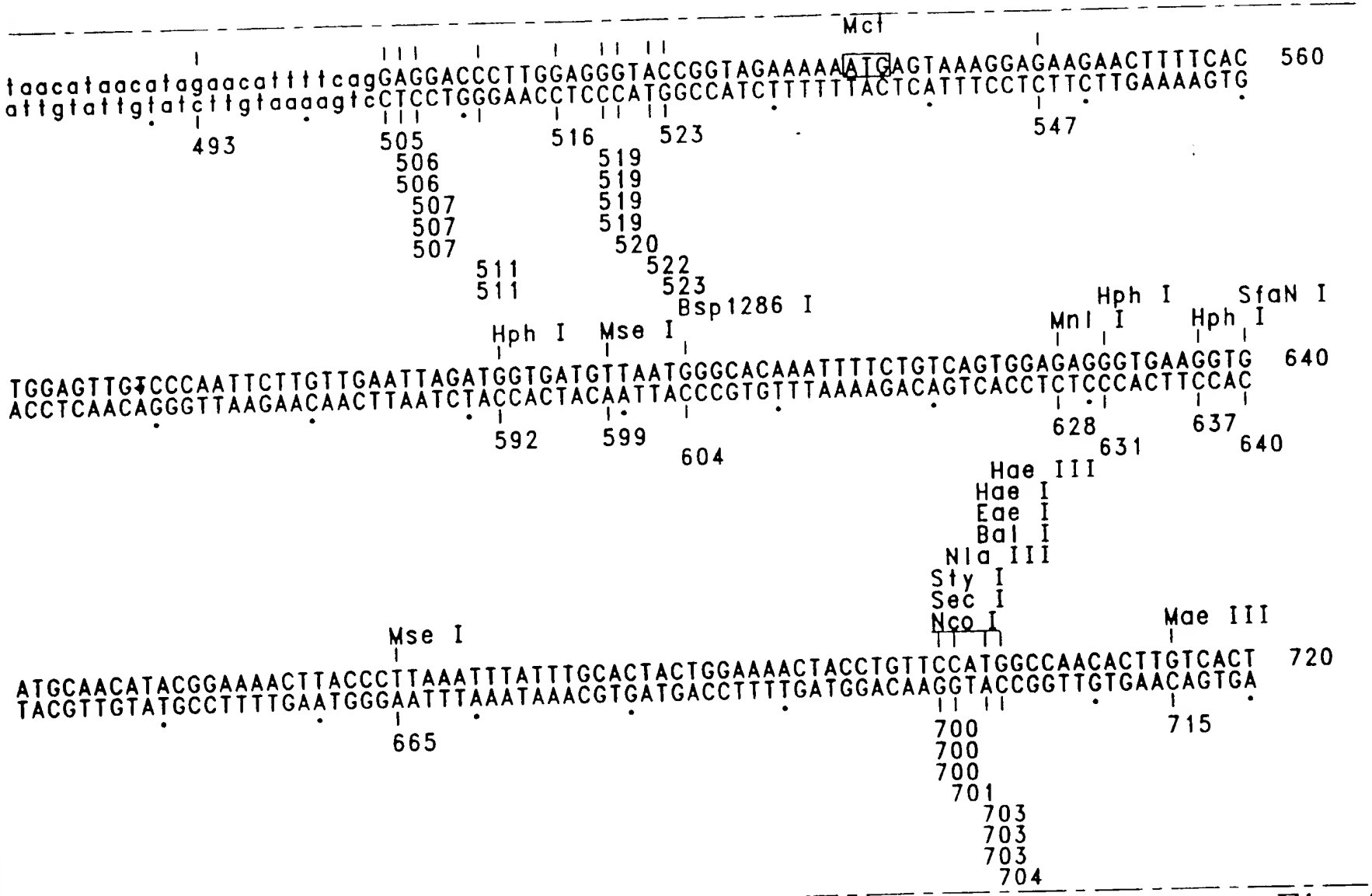
Fig. 7C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Bsp1286 I
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Mse I
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SfaN I
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Hae III
Hae I
Eae I
Bal I
Nla III
Sty I
Sec I
Nco I
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Mae III
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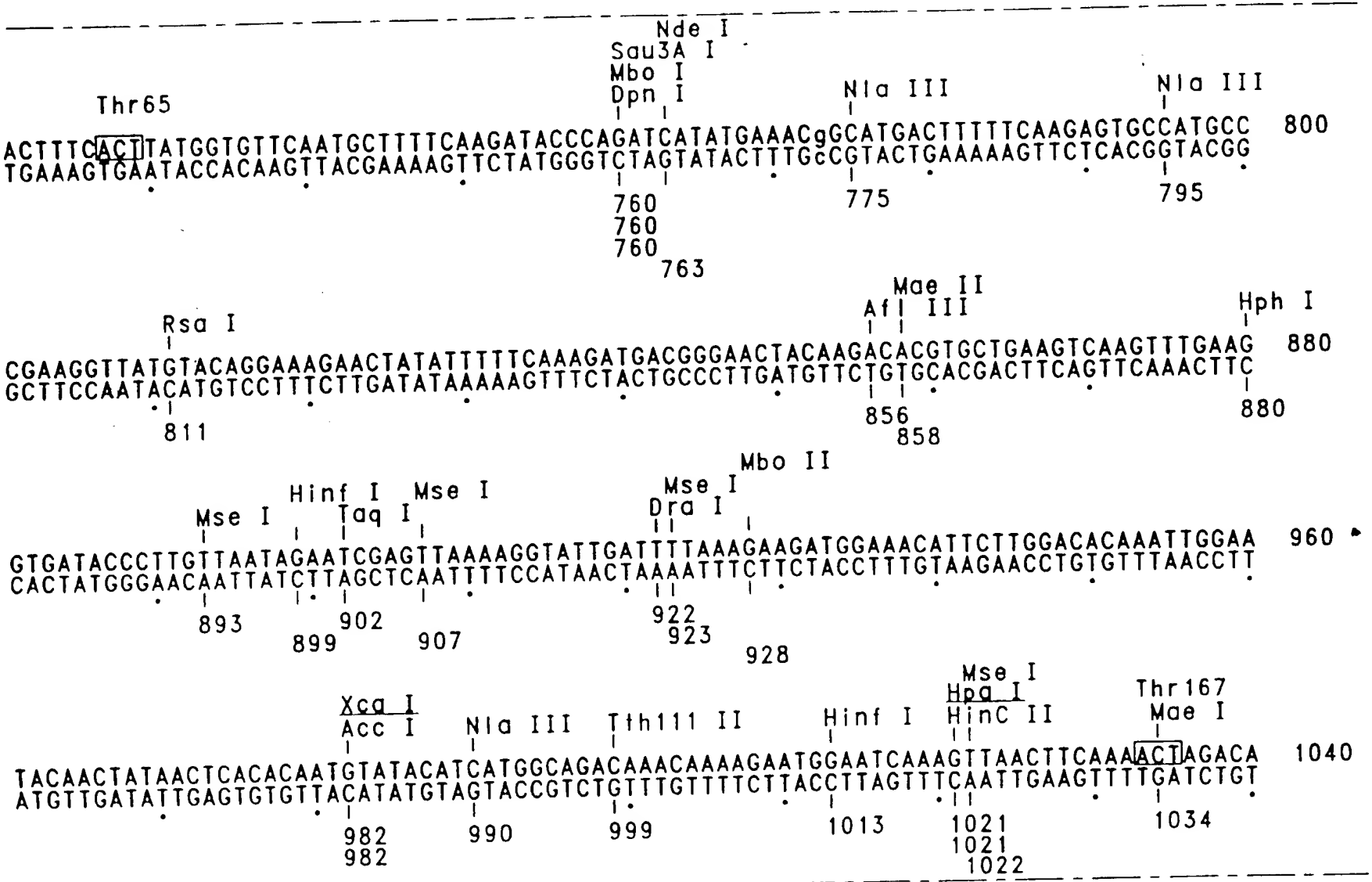
Fig. 7D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Dpn I
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Nla III
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760
760
760
763
775
795
Mae II
Af| III
Rsa I
Hph I
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Hinf I
Mse I
Mse I
Mse I
Taq I
Dra I
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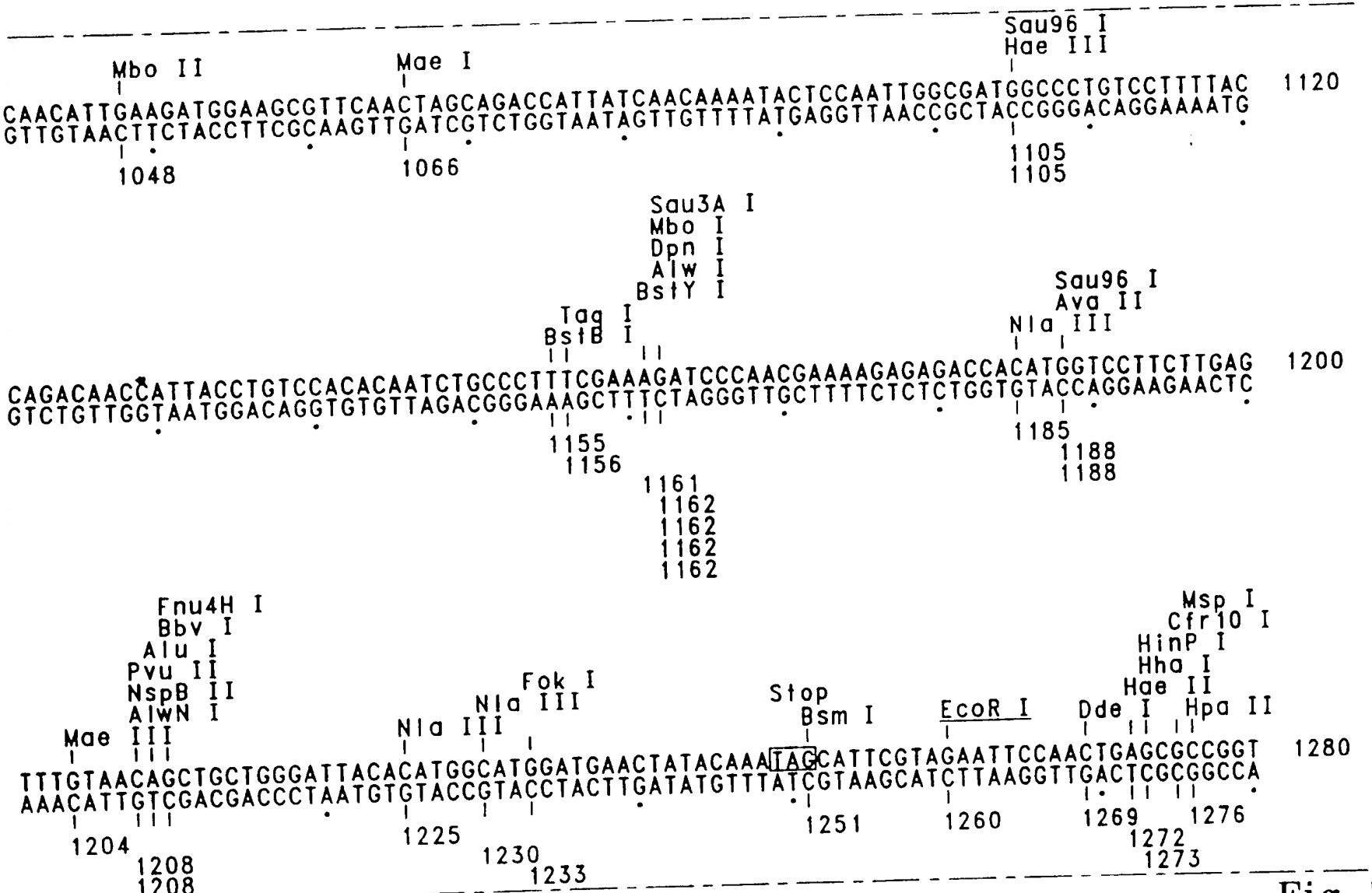
Fig. 7E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Mbo II
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Mbo I
Dpn I
Alw I
BstY I
Tag I
BstB I
Sau96 I
Ava II
Nla III
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Bbv I
Alu I
Pvu II
NspB II
AlwN I
Mae III
Fok I
Nla III
Nla III
Stop
Bsm I
EcoR I
Msp I
Cfr10 I
HinP I
Hha I
Hae II
Dde I
Hpa II
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Fig. 7F

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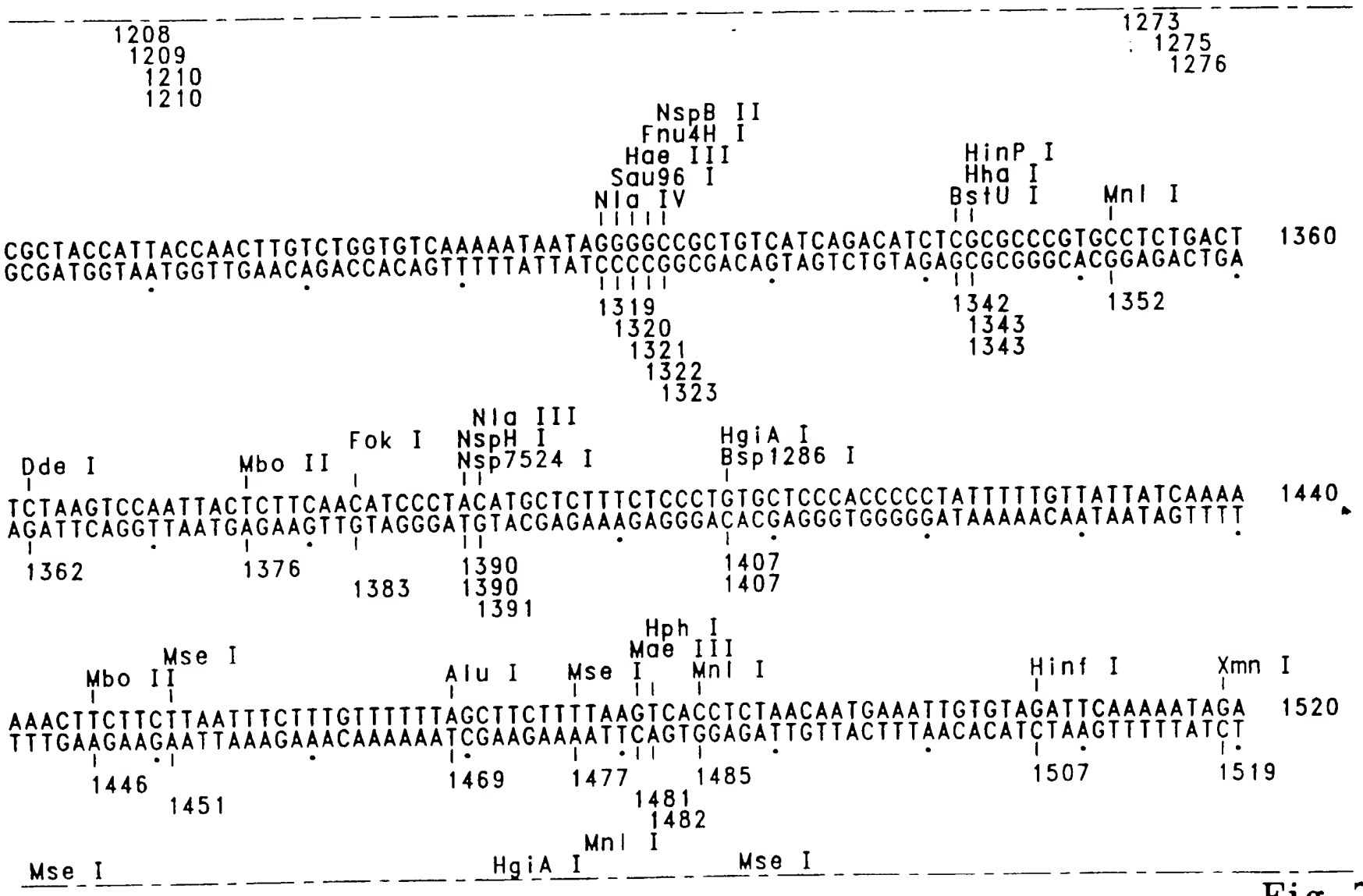
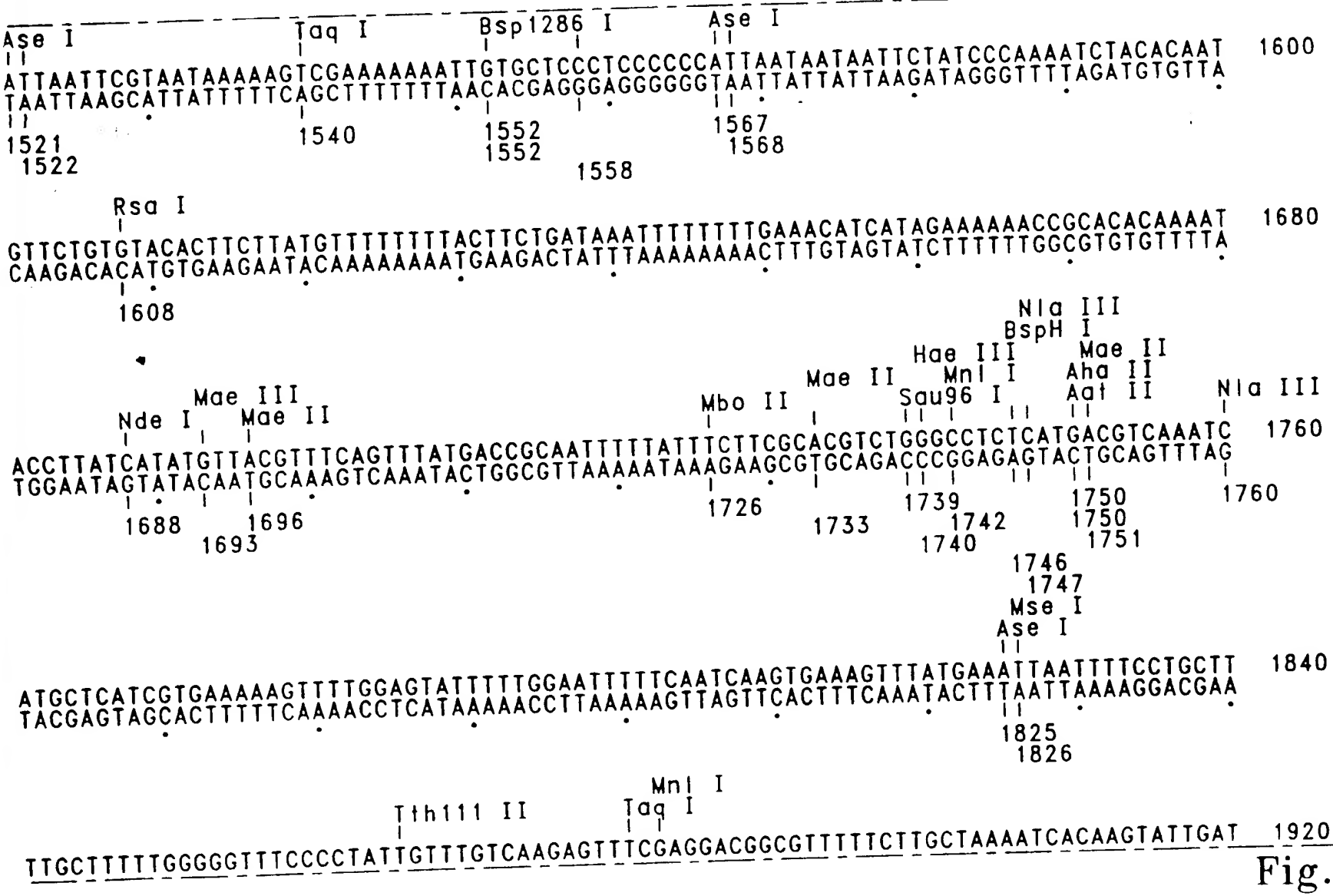


Fig. 7G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Hae III
Mae II
Mae II
Mnl I
Aha II
Aat II
Nla III
Mae III
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Mae II
Mbo II
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Ase I
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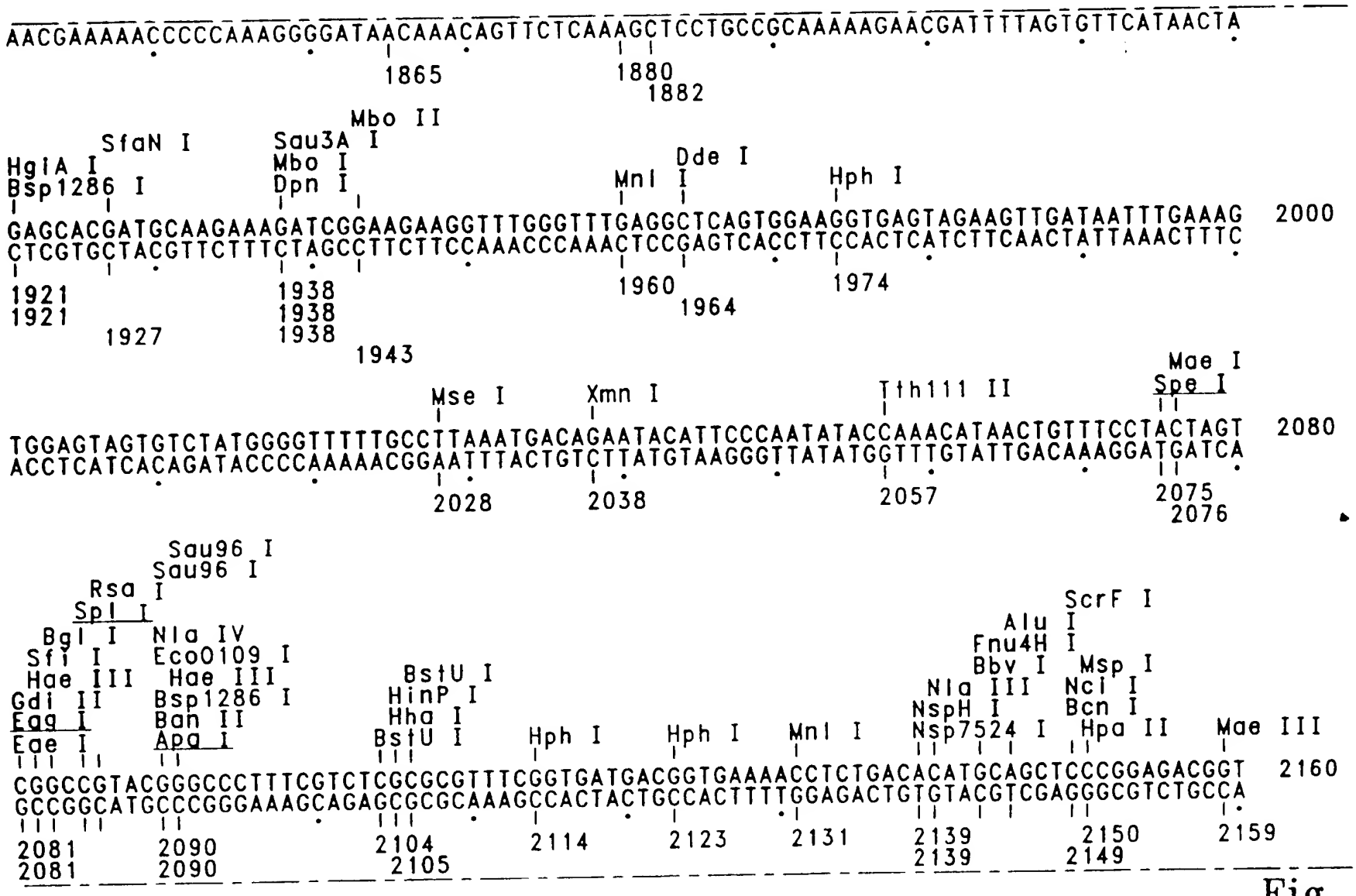
Fig. 7H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Sau3A I
HgiA I
Mbo I
Dde I
Bsp1286 I
Dpn I
MnI I
Hph I
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1974
1921
1938
1964
1927
1938
1943
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Mse I
Xmn I
Tth111 II
Spe I
TGGAGTAGTGTCTATGGGGTTTTTGCCTTAAATGACAGAATACATTCCCAATATACCAAACATAACTGTTTCCTACTAGT 2080
ACCTCATCACAGATACCCCAAAAACGGAATTTACTGTCTTATGTAAGGGTTATATGGTTTGTATTGACAAAGGATGATCA
2028
2038
2057
2075
2076
Sau96 I
Sau96 I
Rsa I
Spl I
ScrF I
Alu I
Bgl I
Nla IV
Fnu4H I
Sfi I
Eco0109 I
Bbv I
Msp I
Hae III
Hae III
BstU I
Nla III
Nci I
Gdi II
Bsp1286 I
HinP I
NspH I
Bcn I
Eag I
Ban II
Hha I
Nsp7524 I
Hpa II
Mae III
Eae I
Apa I
BstU I
Hph I
Hph I
MnI I
CGGCCGTACGGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGT 2160
GCCGGCATGCCCGGGAAAGCAGAGCGCGCAAAGCCACTACTGCCACTTTTGGAGACTGTGTACGTCGAGGGCGTCTGCCA
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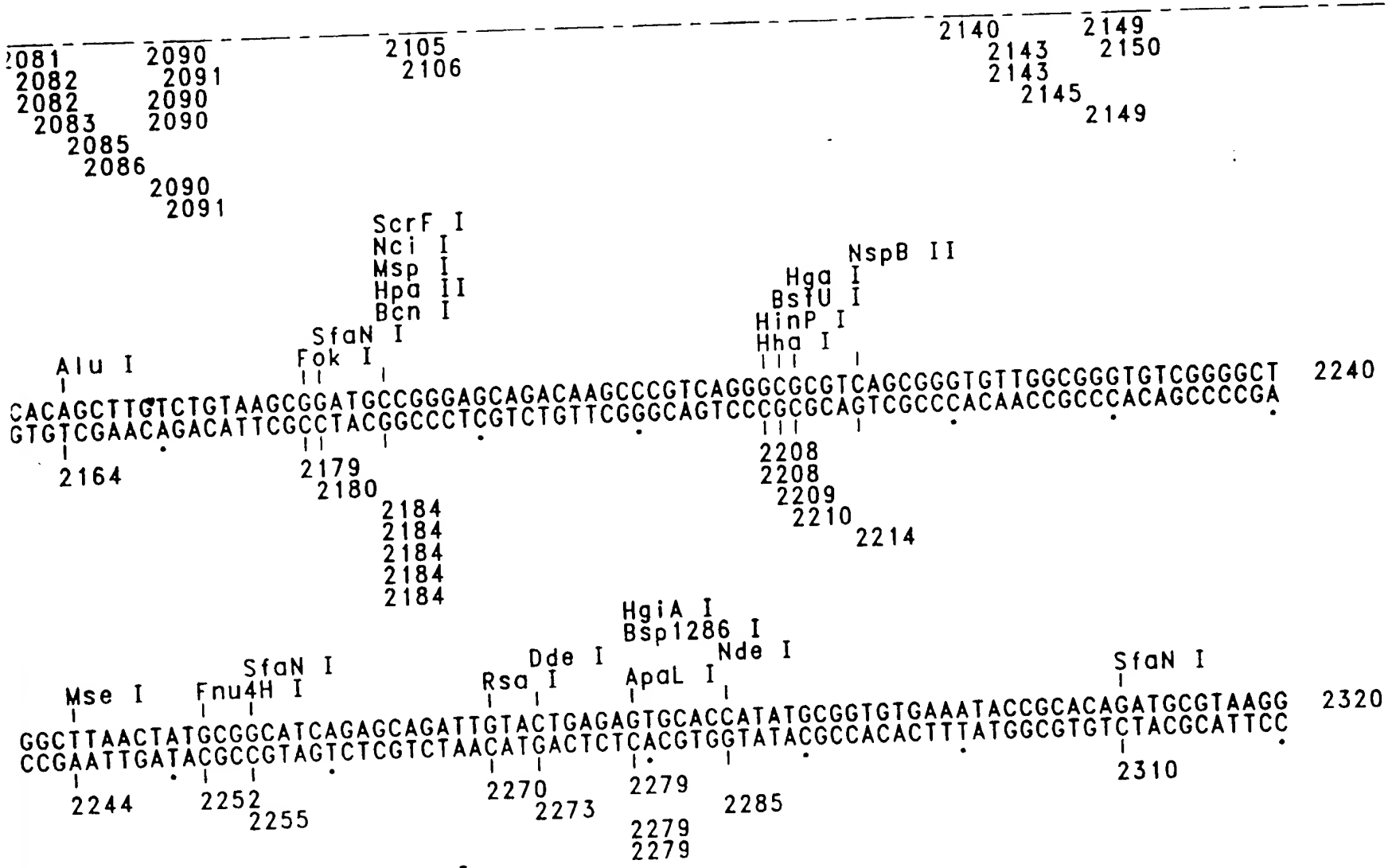
2081
2090

Fig. 7I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2106
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2143
2145
2149
2149
2150
ScrF I
Nci I
Msp I
Hpa II
Bcn I
SfaN I
Fok I
Alu I
NspB II
Hga I
BstU I
HinP I
Hha I
CACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCT 2240
GTGTCGAACAGACATTCGCCTACGGCCCTCGTCTGTTCGGGCAGTCCCGCGCAGTCGCCCACAACCGCCCACAGCCCCGA
2164
2179
2180
2184
2184
2184
2184
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2208
2208
2209
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2214
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Bsp1286 I
Dde I
Nde I
SfaN I
SfaN I
Mse I
Fnu4H I
Rsa I
ApaL I
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CCGAATTGATACGCCGTAGTCTCGTCTAACATGACTCTCACGTGGTATACGCCACACTTTATGGCGTGTCTACGCATTCC
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2285
2310
Mse I
Afl II

Fig. 7J

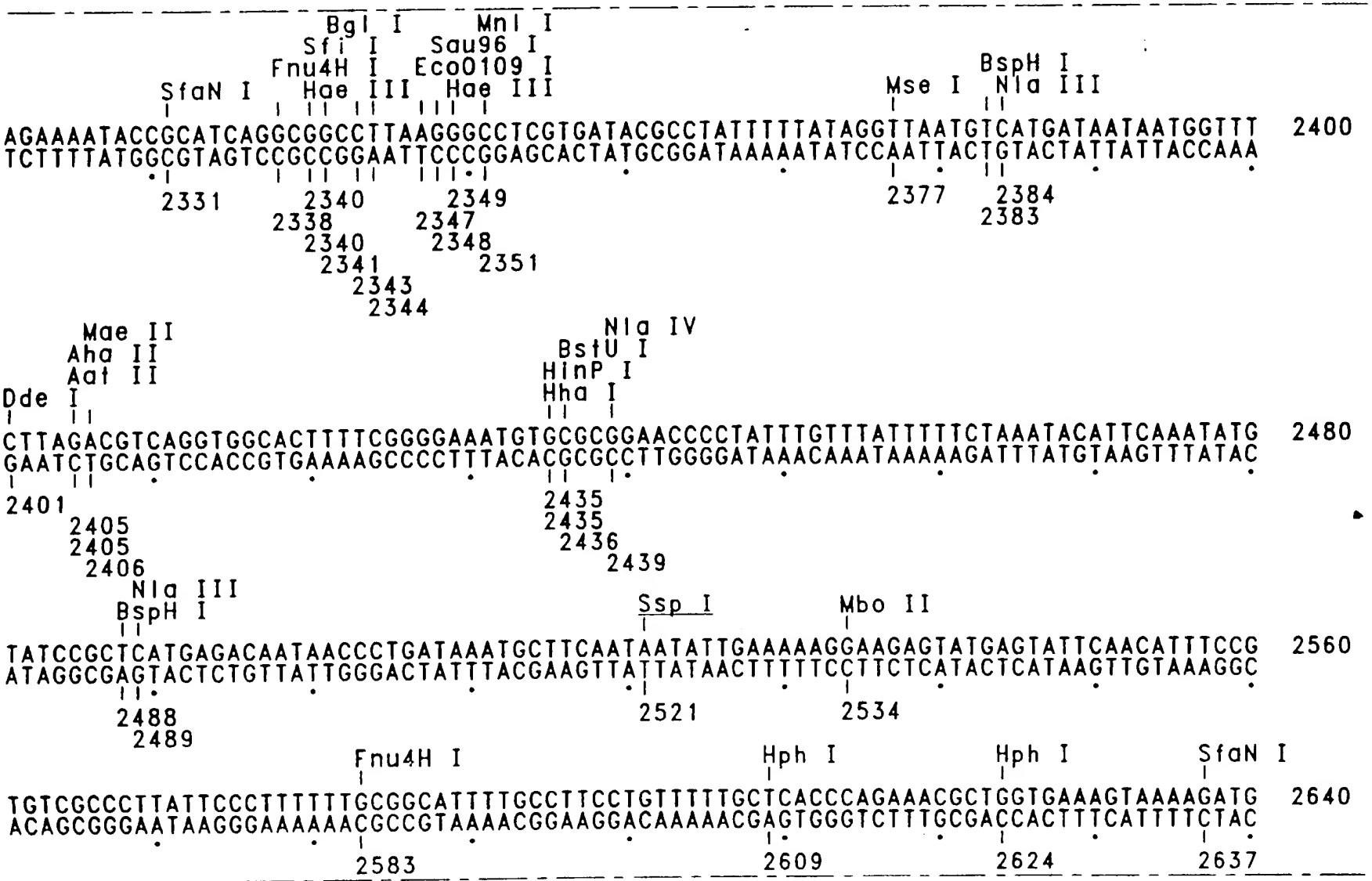
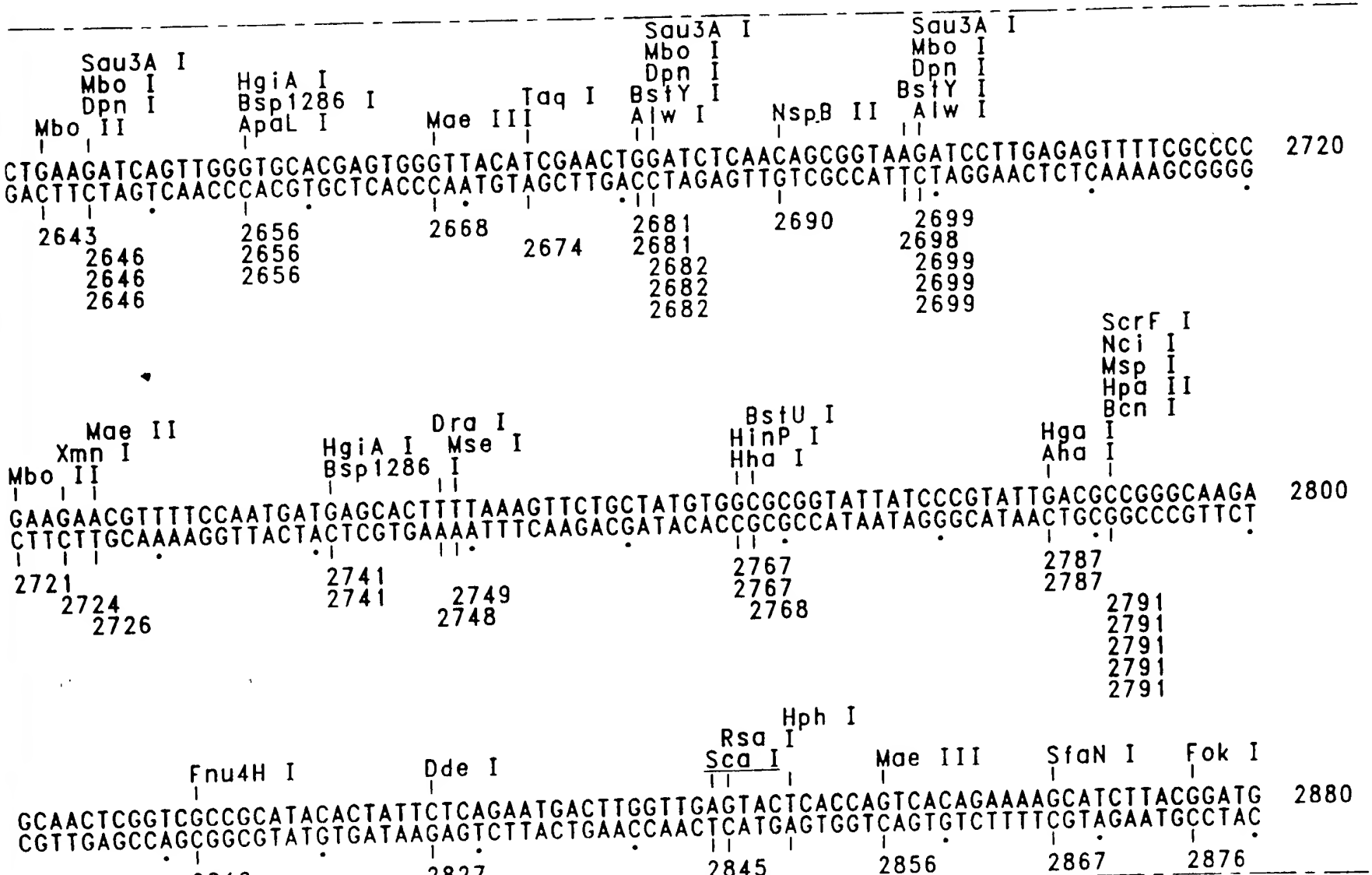


Fig. 7K

SUBSTITUTE SHEET (RULE 26)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Dpn I
HgiA I
Bsp1286 I
ApaL I
Taq I
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Mbo I
Dpn I
BstY I
AIw I
NspB II
Sau3A I
Mbo I
Dpn I
BstY I
AIw I
Mbo II
Mae III
CTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCC 2720
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ScrF I
Nci I
Msp I
Hpa II
Bcn I
Mae II
Xmn I
Mbo II
Dra I
HgiA I
Bsp1286 I
Mse I
BstU I
HinP I
Hha I
Hga I
Aha I
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Hph I
Rsa I
Sca I
Fnu4H I
Dde I
Mae III
SfaN I
Fok I
GCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATG 2880
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Fig. 7L

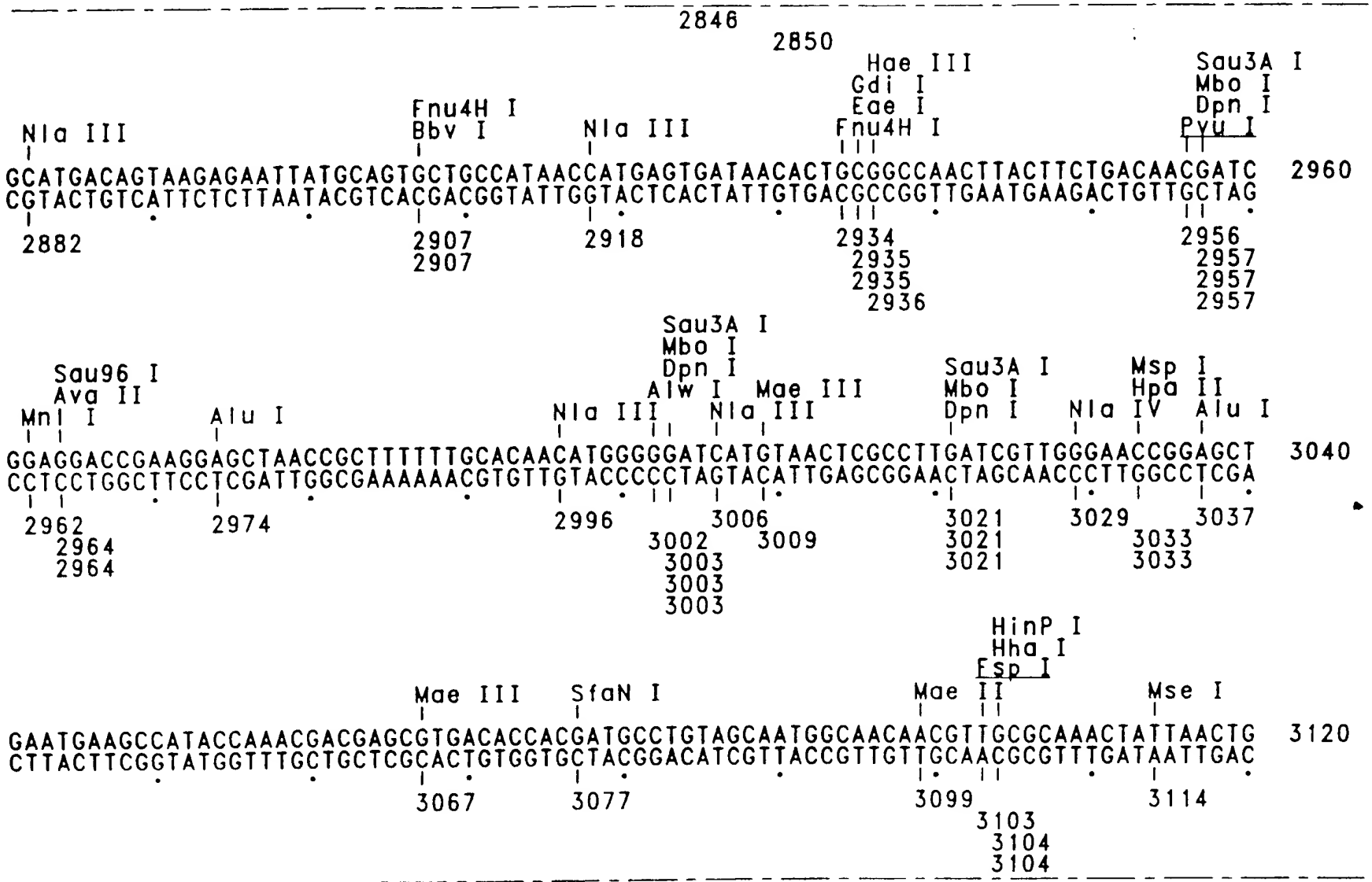

Fig. 7M

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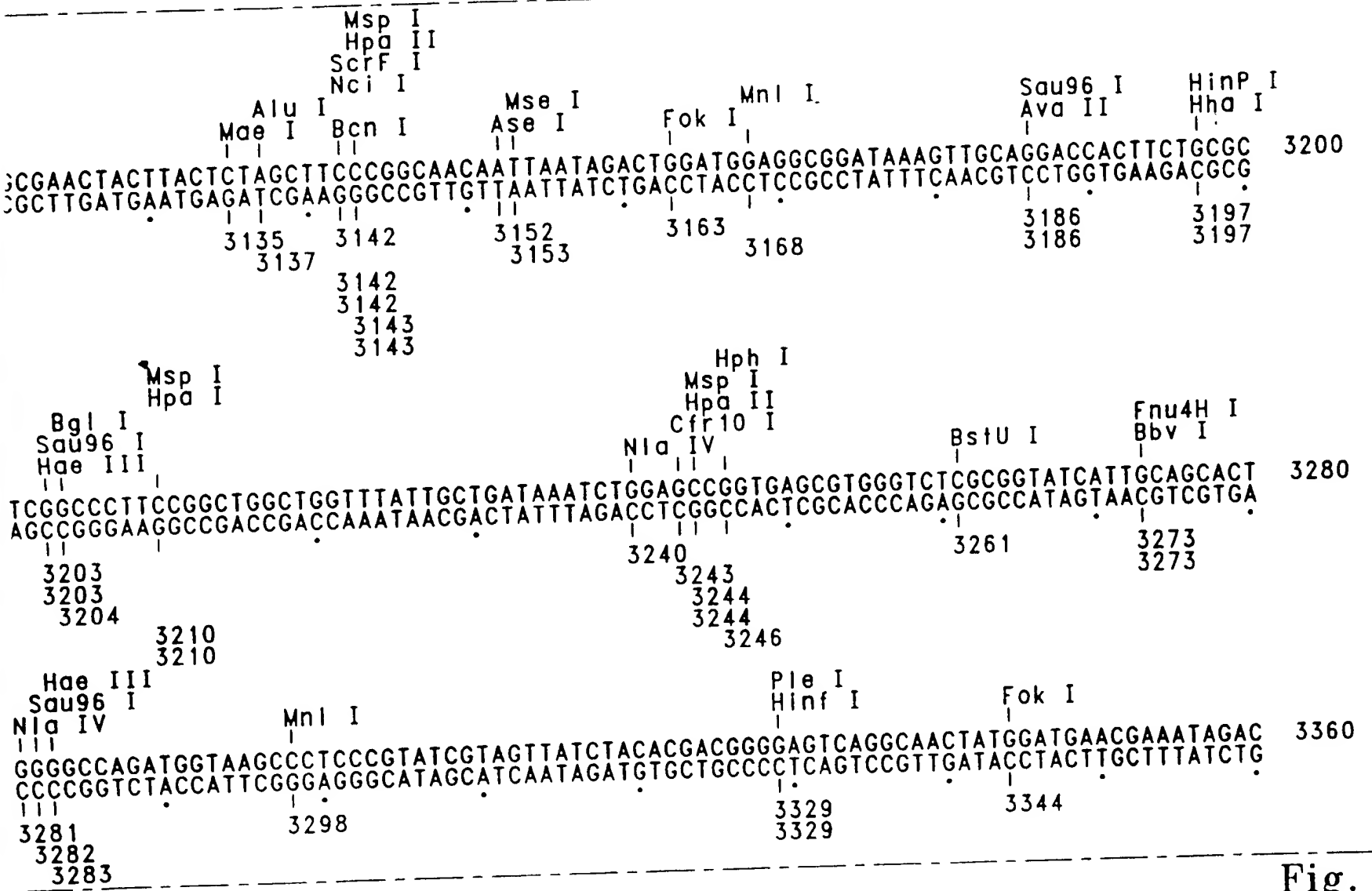
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Ava II
HinP I
Hha I
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3168
3186
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Msp I
Hpa II
Cfr10 I
Bgl I
Sau96 I
Hae III
Nla IV
BstU I
Fnu4H I
Bbv I
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Hae III
Sau96 I
Nla IV
Mnl I
Ple I
Hinf I
Fok I
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3329
3344

Fig. 7N

SUBSTITUTE SHEET (RULE 26)

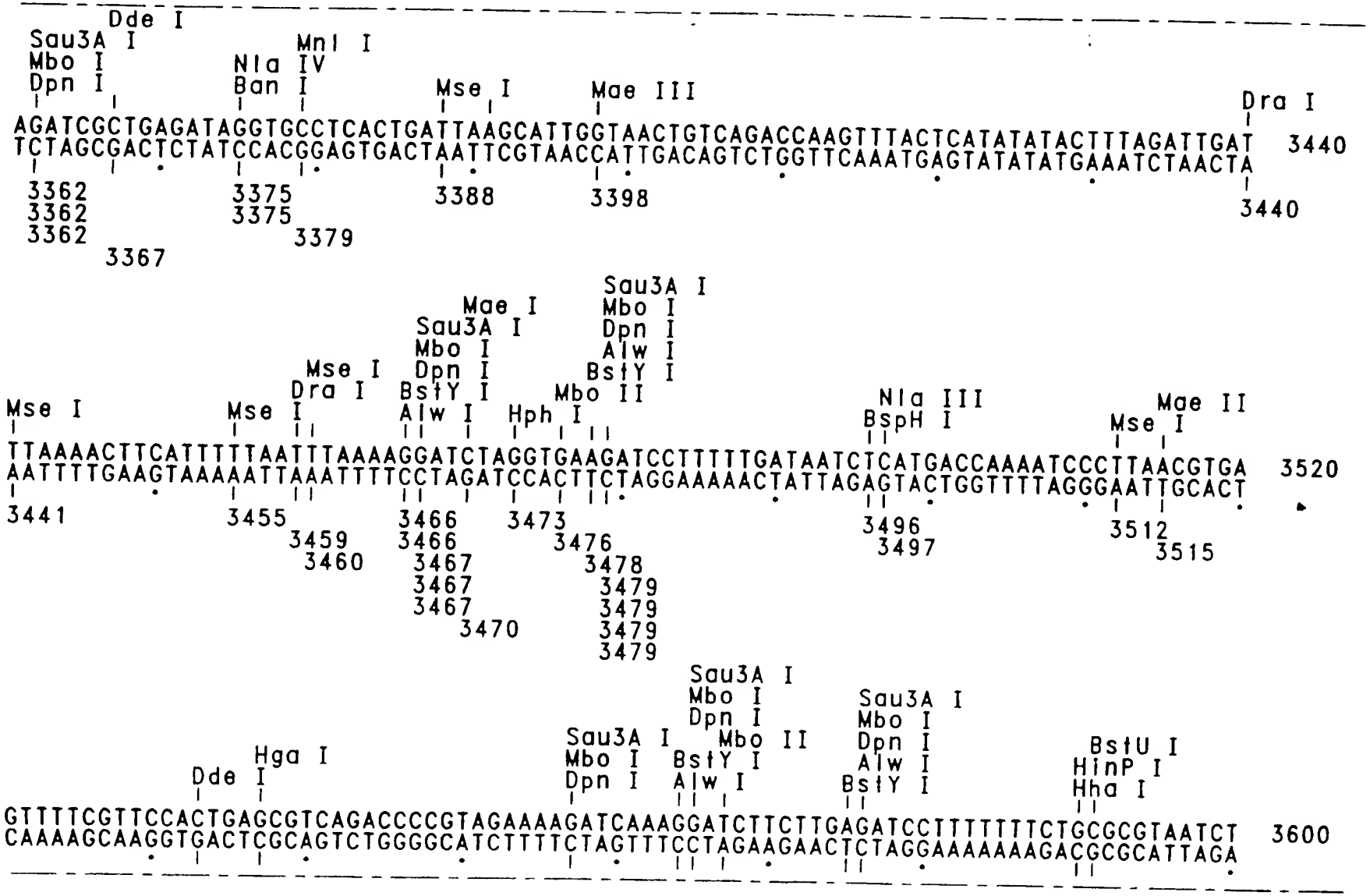
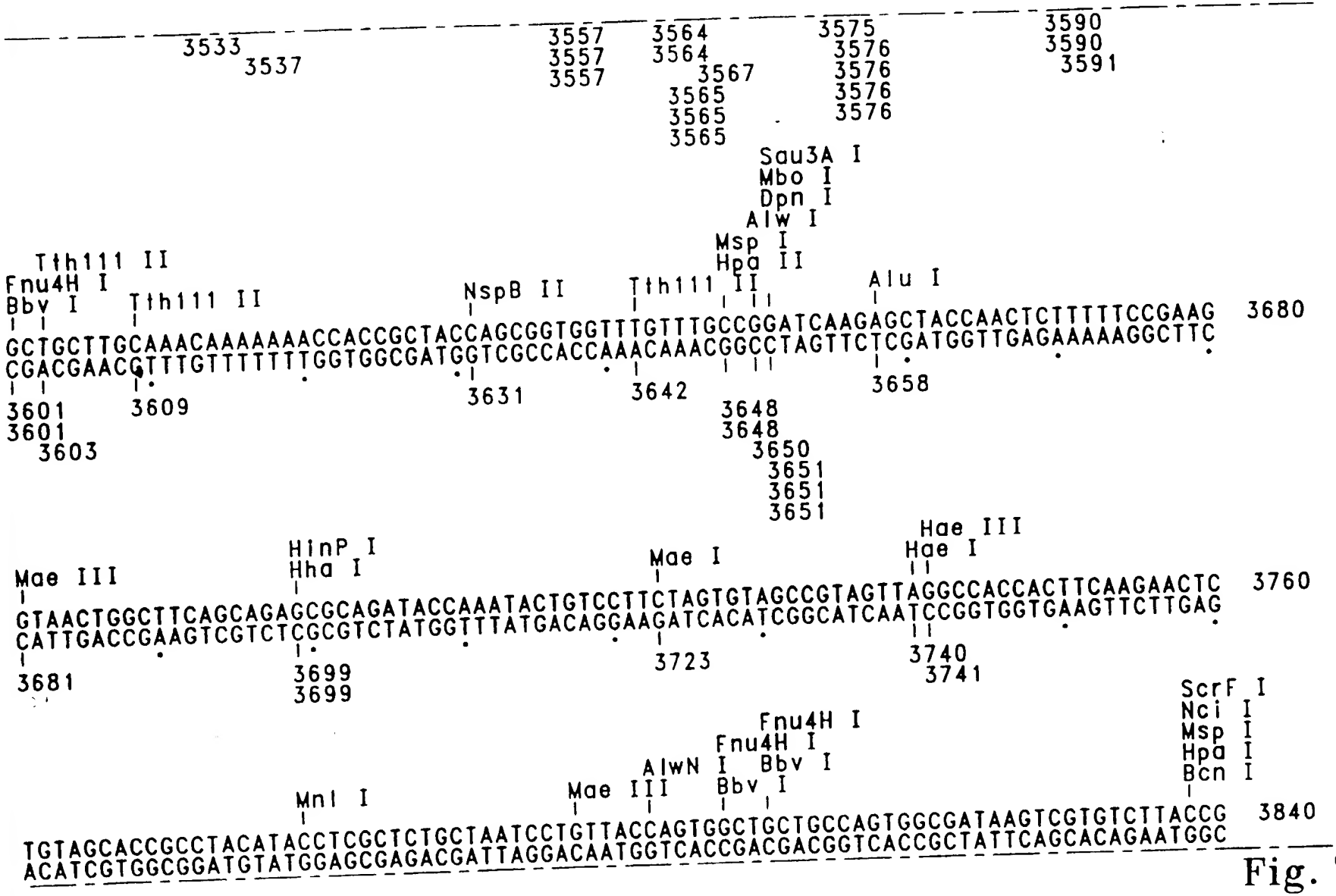


Fig. 70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Mbo I
Dpn I
Alw I
Msp I
Hpa II
Tth111 II
Fnu4H I
Bbv I
Tth111 II
NspB II
Tth111 II
Alu I
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3651
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Hae III
HinP I
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Mae I
Hae I
Mae III
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CATTGACCGAAGTCGTCTCGCGTCTATGGTTTATGACAGGAAGATCACATCGGCATCAATCCGGTGGTGAAGTTCTTGAG
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3740
3741
ScrF I
Nci I
Msp I
Hpa I
Bcn I
Fnu4H I
Fnu4H I
AlwN I
Bbv I
Mae III
Bbv I
Mnl I
TGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCG 3840
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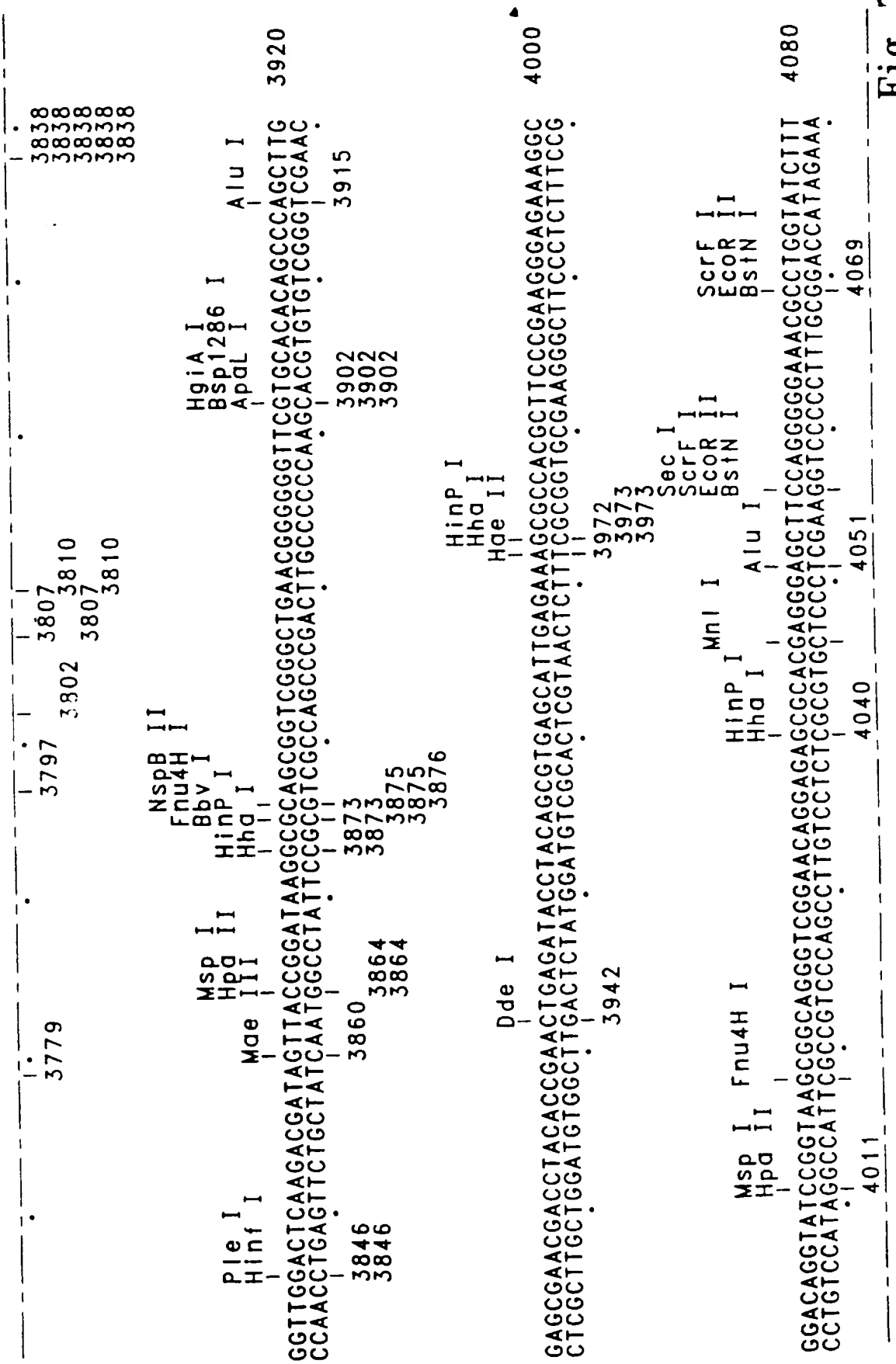
Fig. 7P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Bbv I
HinP I
Hha I
Msp I
Hpa II
Mae III
Ple I
Hinf I
3846
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3860
3864
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3873
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3875
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ApaL I
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Hae II
Dde I
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3973
Sec I
ScrF I
EcoR II
BstN I
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Alu I
ScrF I
EcoR II
BstN I
Msp I
Hpa II
Fnu4H I
4011
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GAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGC
CTCGCTTGCTGGATGTGGCTTGACTCTATGGATGTCGCACTCGTAACTCTTTCGCGGTGCGAAGGGCTTCCCTCTTTCCG
GGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTT
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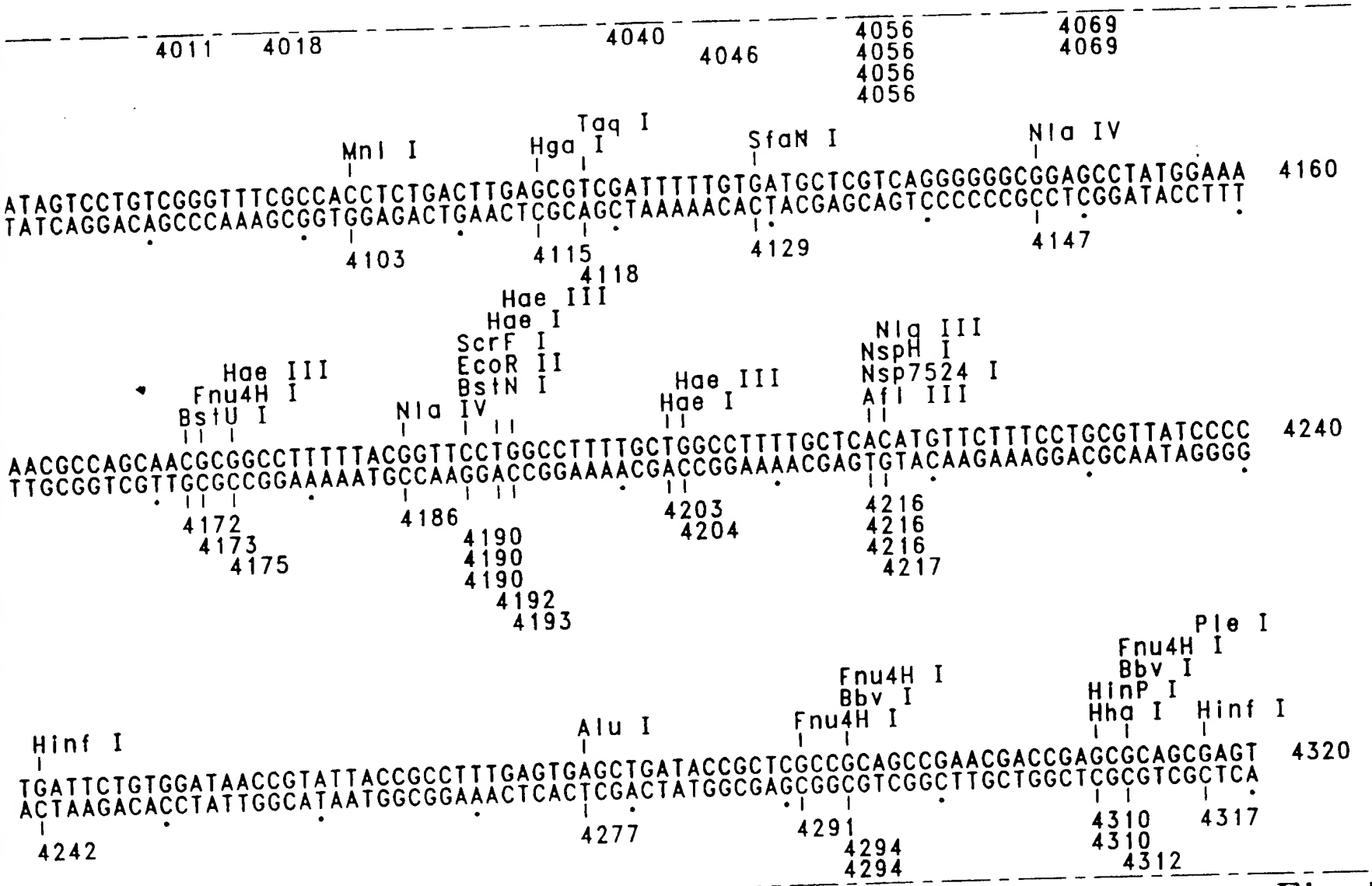
Fig. 7Q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Hga I
SfaN I
NIa IV
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4103
4115
4118
4129
4147
Hae III
Hae I
ScrF I
EcoR II
BstN I
Hae III
Fnu4H I
BstU I
NIa IV
Hae III
Hae I
NIa III
NspH I
Nsp7524 I
AfI III
AACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCC 4240
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4217
Ple I
Fnu4H I
Bbv I
Fnu4H I
Bbv I
HinP I
Hha I
Hinf I
Hinf I
Alu I
Fnu4H I
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ACTAAGACACCTATTGGCATAATGGCGGAAACTCACTCGACTATGGCGAGCGGCGTCGGCTTGCTGGCTCGCGTCGCTCA
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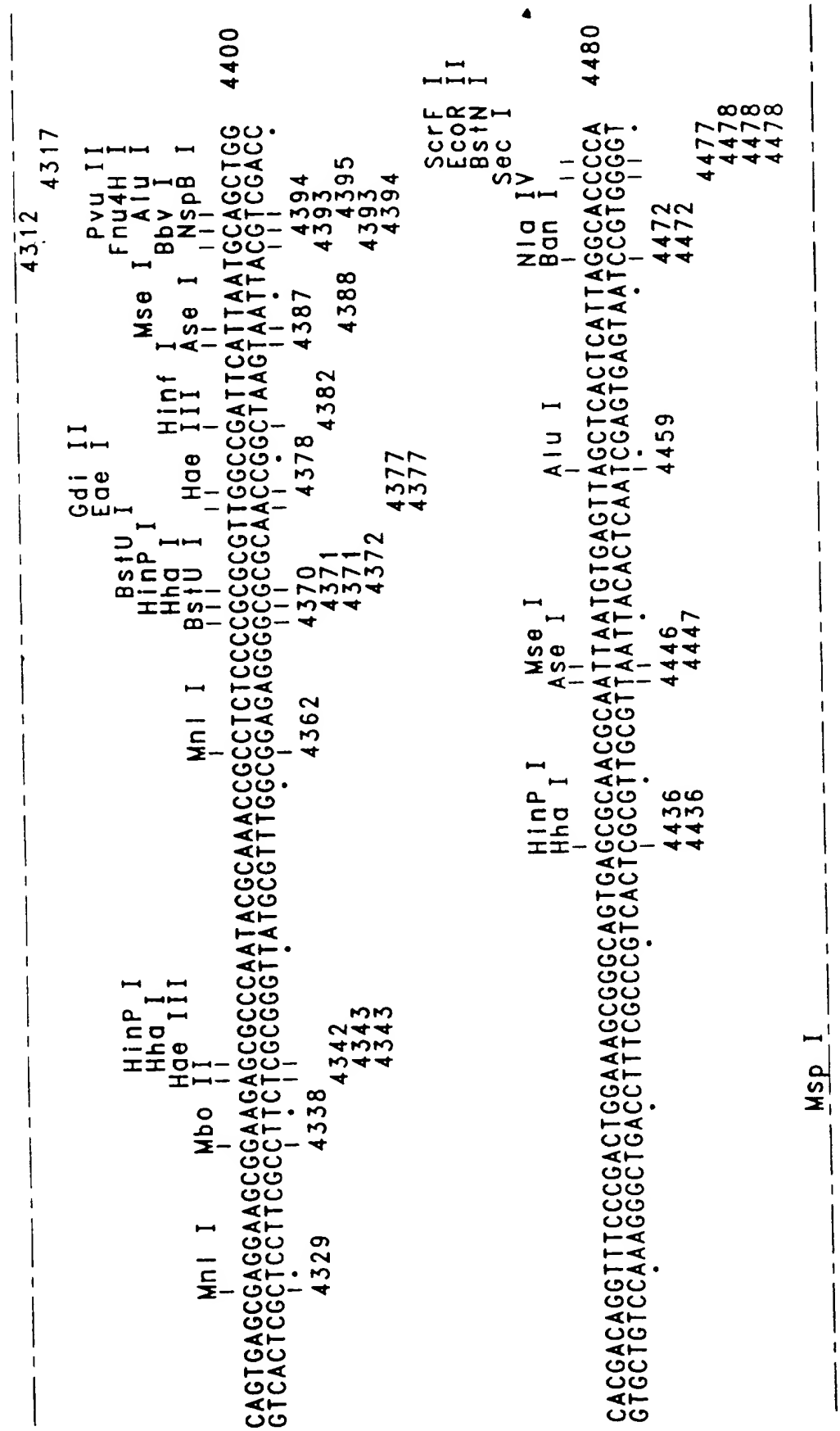
Fig. 7R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Pvu II
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Alu I
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Hha I
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Hinf I
Hae III
BstU I
Ase I
NspB I
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ScrF I
EcoR II
BstN I
Sec I
Nla IV
Ban I
4480
HinP I
Hha I
Hae III
Mbo II
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Mnl I
Mse I
Ase I
Alu I
HinP I
Hha I
Msp I
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4478

Fig. 7S

```
                    Hpa II                                                 Alu I
                    |                                                      |
GGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATG  4560
CCGAAATGTGAAATACGAAGGCCGAGCATACAACACACCTTAACACTCGCCTATTGTTAAAGTGTGTCCTTTGTCGATAC
                    |                                                      |
                    4500                                                   4554
                    4500

 Nla III
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ACCATGATTACGCC  4574
TGGTACTAATGCGG
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 4563
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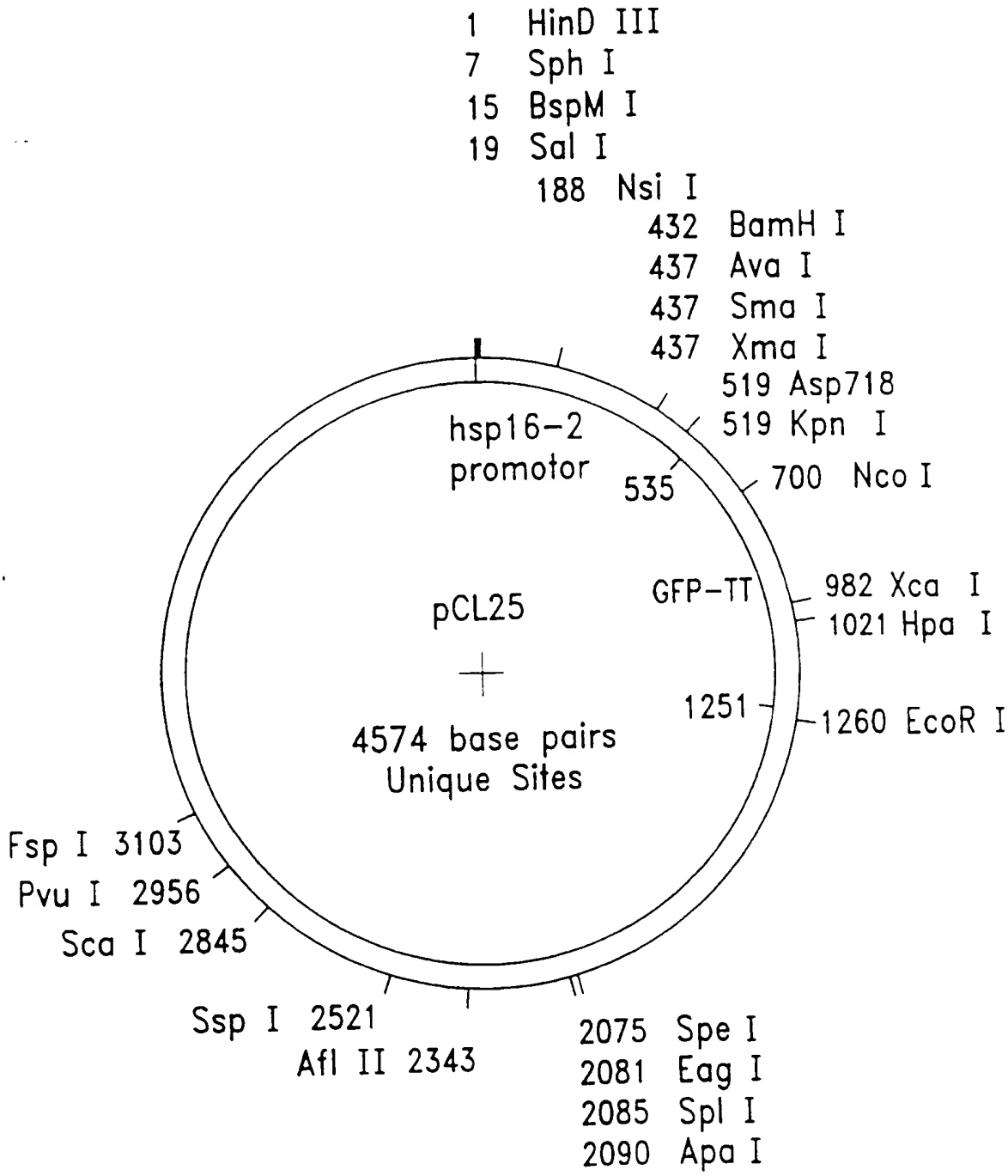
Fig. 7T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7 Sph I
15 BspM I
19 Sal I
188 Nsi I
432 BamH I
437 Ava I
437 Sma I
437 Xma I
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519 Kpn I
700 Nco I
982 Xca I
1021 Hpa I
1260 EcoR I
1251
535
hsp16-2
promotor
GFP-TT
pCL25
4574 base pairs
Unique Sites
Fsp I 3103
Pvu I 2956
Sca I 2845
Ssp I 2521
Afl II 2343
2075 Spe I
2081 Eag I
2085 Spl I
2090 Apa I

Fig. 8

```
   1  atcacaatga catccttatg tatatgcatt ctttttattg tttcctgata tttattctct
  61  ctttatcatt tcacttttte ttggcactcg tccaaaaatc tcaattaaaa atttctggat
 121  atatctttag atctaactga aaatttccag atgaccctaa ctacggcgac gtccggcgcc
 181  attgtatttt ctggagccac tttgttggtt tctcttttg ccgctgcttc gctttacagt
 241  caagtttcca atatctggaa tgagctggat gccgaaatcg ccaacttcag agtgagtttc
 301  ctatatagca tttctaattc actgataatt tcaattattt agagtctcac tgaagacatg
 361  tgggttgata tggttaaact tggagcagga accgcttcca accgtgtgag acgtcaacaa
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 481  ggatatggag caagccagcc agctccacca gagaaattcc cagatggtat accaaatgga
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 661  gctggaccag aaggagagga aggaccagat gggcacgatg gacaagacgg agtcccagga
 721  tttgacggaa aagatgccga agatgttcaa aacaccccac caacaggatg cttcacctgt
 781  ccacaaggac cacttggacc acaaggacca aatggagctc caggactcag aggaatgcgc
 841  ggagctcgtg gacaaccagg acgtccagga agagacggaa acccaggaat gccaggagac
 901  tgtggaccac caggggcccc aggatccgat ggaaagccag gatctccagg aggaaaggga
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```

Fig. 9

MTLTTATSGAIVFSGATLLVSLFAAASLYSQVSNIWNELDAEIANFRSLTEDMWVDMVK
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KFPGGGFPDGPFPNGGGPRGGNQCQCTVENSCPPGPAGPEGEEGPDGHDGQDGVPGFD
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DCGPPGAPGSDGKPGSPGGKGDDGERPLGRPGPRGPPGEAGPEGPQGPTGRDAYPGQSG
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Fig. 10

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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A3

(11) International Publication Number: WO 98/28971

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(21) International Application Number: PCT/US97/23819

(22) International Filing Date: 31 December 1997 (31.12.97)

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60/080,222	3 January 1997 (03.01.97)	US

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(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: LINK, Christopher [US/US]; 1837 Spruce Street, Boulder, CO 80302 (US).

(74) Agents: MONROY, Gladys, H. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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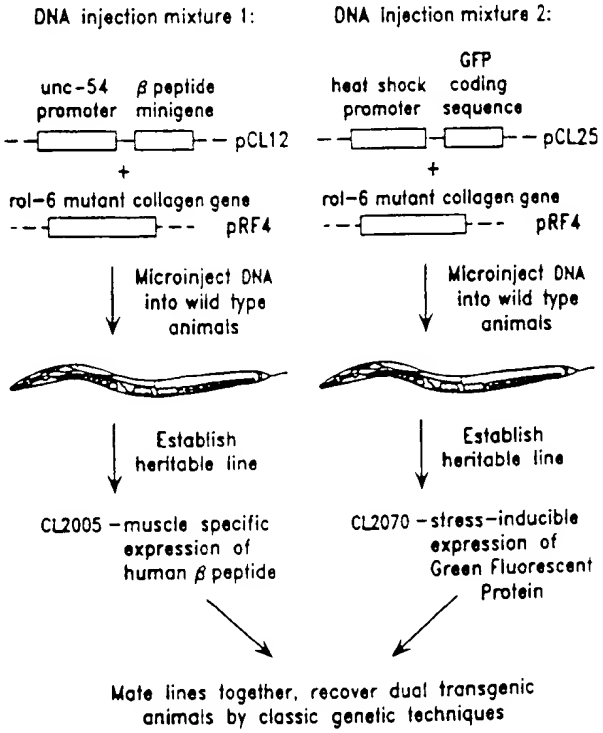
(54) Title: TRANSGENIC ANIMALS FOR *IN VIVO* VISUALIZATION OF BETA-AMYLOID TOXICITY

(57) Abstract

The present invention relates to methods and compositions for visualization of the toxic effects of transgenes *in vivo*. In particular, the present invention provides methods and compositions for the production and use of transgenic, including dually transgenic, *Caenorhabditis elegans* for visualization of the toxic effects of β-amyloid accumulation *in vivo*.



INTERNATIONAL SEARCH REPORT

Ir ational Application No
PCT/US 97/23819

A CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/00 A01K67/033 C07K14/435 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA 2 088 379 A (CANDIDO EDWARD P M ;STRINGHAM EVE G (CA); JONES DONALD (CA)) 30 July 1994	1-4,6,7, 13-15,20
Y	see the whole document	1-23
Y	LINK, C.D.: "Expression of human beta-amyloid peptide in transgenic Caenorhabditis elegans" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 92, no. 20, 26 September 1995, pages 9368-9372, XP002065639 WASHINGTON US cited in the application	1-8, 10-15, 18-20, 22,23
X	see the whole document	11-15

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[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

Special categories of cited documents

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

& document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 September 1998	11 09 1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl Fax (+31-70) 340-3016	Chambonnet, F

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/23819

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
Y	LINK, C.D.. "Transgenic Caenorhabditis elegans as model system to study amyloid formation and toxicity" NEUROBIOLOGY OF AGING. vol. 15, no. suppl. 1, 29 July 1994, page S33.XP002065640 see the whole document	1,5-7
X	WO 95 11968 A (ATHENA NEUROSCIENCES INC ;LILLY CO ELI (US)) 4 May 1995 see page 38, line 3 - page 39, line 22	11,12,19
Y	WO 93 23533 A (TSI CORP) 25 November 1993 see page 27, paragraph 3 - page 28, paragraph 2	11-15
Y	CHALFIE M ET AL: "GREEN FLUORESCENT PROTEIN AS A MARKER FOR GENE EXPRESSION" SCIENCE. vol. 263, no. 5148, 11 February 1994, pages 802-805, XP002003599 see the whole document	1,2,6,7, 9,11, 13-17, 20,21
Y	MOSS, J. B. ET AL.. "Green fluorescent protein marks skeletal muscle in murine cell lines and zebrafish" GENE. vol. 173, no. 1, 1996, pages 89-98, XP002065641 AMSTERDAM NL see the whole document	1,2,9, 13,16,17

INTERNATIONAL SEARCH REPORT

Int. ..tional application No
PCT/US 97/23819

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons

1 ☐ Claims Nos
because they relate to subject matter not required to be searched by this Authority, namely

2. ☐ Claims Nos
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically

3 ☐ Claims Nos
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6 4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows

see additional sheet

1 [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee

3 ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos

4 ☐ No required additional search fees were timely paid by the applicant Consequently, this International Search Report is restricted to the invention first mentioned in the claims, it is covered by claims Nos

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest

[X] No protest accompanied the payment of additional search fees

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-10 and partially 11-23

a transparent animal comprising :
(a) a toxic transgene;
(b) an inducible promotor operably linked to a reporter gene; wherein the toxic transgene induces the promotor and wherein the expression of the reporter gene is detectable in vivo; and
a method for providing such a dually transgenic transparent non-human animal comprising :

a) providing :
i) a first and a second non-human transparent animals;
ii) a first toxic transgene; and
iii) a second transgene, comprising an inducible promotor operably linked to a reporter gene, wherein the toxic transgene induces the promotor and wherein the expression of the reporter gene is detectable in vivo,

b) introducing said first toxic transgene into the genome of said first non-human animal to produce a first transgenic animal and introducing said second transgene into the genome of said second non-human animal to produce a second transgenic animal; and

c) mating said first transgenic animal with said second transgenic animal to produce a dually transgenic transparent non-human animal wherein said toxic transgene is expressed and induces the promotor so that the said reporter gene is also expressed and detectable in vivo;

2. Claims: partially 11-23

a method to produce a dually transgenic non-human animal with a toxic transgene and a reporter are expressed comprising :

a) providing :
i) a first and a second non-human animal;
ii) a first toxic transgene; and
iii) a second transgene, comprising a reporter,

b) introducing said first toxic transgene into the genome of said first non-human animal to produce a first transgenic animal and introducing said second transgene into the genome of said second non-human animal to produce a second transgenic animal; and

c) mating said first transgenic animal with said second transgenic animal to produce a dually transgenic non-human animal wherein said toxic transgene and said reporter are expressed

in the cases non relevant of the first described subject, that means where it is not a transparent animal comprising :

(a) a toxic transgene;
(b) an inducible promotor operably linked to a reporter gene; wherein the toxic transgene induces the promotor and wherein the expression of the reporter gene is detectable in vivo.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/23819

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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